Exploring the role of dopamine neurons in salt intake

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Declaration of originality

The experiments described within this thesis are my own unaided work, except where explicitly stated. No part of this thesis has been submitted for any other degree or professional qualification.

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Abstract

Sodium is an essential element, but in the modern world average salt intake is too high and this is associated with a significant disease burden. In most mammals, depletion of salt leads to a profound behavioural change. This is termed salt appetite. The components of salt appetite are motivation for, ingestion of and enjoyment for hypertonic saline (which is aversive in the salt replete state). Deciphering the neuronal circuits that drive salt seeking behaviour may enable possible future manipulation of pathological salt appetite. Ventral tegmental area (VTA) dopamine neurons play key roles in motivated behaviours. I therefore hypothesise that the mesolimbic dopamine system is an essential component of the circuits driving salt seeking behaviour. To test this idea I studied the effect that manipulation of dopamine neuron activity has on salt appetite.

My first step required the establishment of a reliable salt appetite in mice. To achieve this I used a combination of low salt feed and furosemide-induced natriuresis. I quantified the appetite for salt by measuring the intake of salt jellies of varying concentrations in a canteen setup. To control the firing rate of the dopamine neurons I have used optogenetics and chemogenetics.

Strikingly, optogenetic excitation of dopamine neurons decreased salt intake in a rapid and reversible manner, despite a strong salt appetite. Importantly, optogenetic stimulation was not aversive, did not induce hyperactivity, and did not interfere with salt concentration preferences when there was no appetite. Optogenetic inhibition, chemogenetic excitation, and chemogenetic inhibition experiments suggested that a 'burst-like' pattern of excitation was important to the reduction in salt intake. In addition, optogenetic excitation of dopamine neurons reduced consumption of sucrose following an overnight fast, indicating a more general role of VTA dopamine neuron excitation in suppressing appetite.

Acknowledgements

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Chapter 1 Introduction

1.1 Clinical and Public Health Importance of salt intake

Hypertension and its cardiovascular, neurological and renal sequelae are major health issues worldwide. The link between high salt intake and hypertension (Intersalt Cooperative Research, 1988) as well as many other diseases, has been established and the benefit of salt reduction has been accepted. There are therefore major public health initiatives to reduce salt intake (WASH; WHO, 2004). Unfortunately, the ability of the public and patients to adhere to a low salt diet is variable. This is illustrated by a review of long term studies which shows that, over at least 5 decades and across 45 countries, human salt intake, as defined by 24 hour urinary sodium excretion (UNaV), is characterised by a narrow range that is remarkably reproducible (McCarron et al., 2013) (Asayama et al., 2014) showing no response to public health drives. The lack of variability also suggests that salt intake is physiologically set.

Plasma sodium concentration is maintained between 135 and 145 mmol/L. This control is vital since hyponatraemia has profound multi-organ consequences which may be fatal. Upon correction there may also be sequelae, including, for example, centre pontine demyelination. Chronic salt depletion, through diet or using low sodium dialysate during dialysis, has been associated with increased mortality (Alderman and Cohen, 2012). It is therefore essential to maintain total body salt. This is normally achieved through physiological control of loss and intake. In certain patient groups (for example the elderly and during diarrhoeal illness) the physiological ability to respond to salt depletion is impaired due to medication or illness. It is therefore important to ensure that intake is adequate.

In the modern world, however, high salt intake has been linked with a greater disease burden than low intake. In addition to the long term consequences of a high salt diet for the general population, some disease states are exacerbated acutely by failure to comply with salt restriction. For example, anuric haemodialysis patients are fluid restricted to limit interdialytic weight gains to 2kg. Salt intake drives osmotic thirst, hence fluid restriction requires salt restriction. Patients who fail to adhere have a worse prognosis in the long term and in the short term may present with life threatening pulmonary oedema. Patients with heart failure conserve salt and water, which exacerbates their symptoms. Fluid restriction, which necessitates salt restriction, is required. Many patients find this very difficult. A recent study that shows patients with heart failure have a higher

salt preference than controls (de Souza et al., 2012) may help to explain why there is so much difficulty in complying with salt restriction.

McCarron *et* al (2013) interpreted their results, which shows no variation in salt intake over 5 decades, to support the concept that salt intake is physiologically set and therefore public health drives to reduce intake are futile. Public health protagonists dispute this analysis (Cappuccio et al., 2013). McCarron *et al*'s results may be misleading since they are based on 24 hour UNaV which is an inaccurate way of assessing salt intake (Lerchl et al., 2015; Titze et al., 2015). Also, whilst there is research to support the homeostatic control of salt intake in a state of physiological need, there is insufficient research with regards to the neural networks that drive salt seeking behaviour and the interplay between environment and this physiological drive. Therefore the assertion that control of salt intake is an inflexible process may not be correct. There is also minimal research regarding the control of salt intake in a need-free state.

1.2 Salt appetite

Sodium is an essential element and its concentration within body compartments is tightly controlled by numerous homeostatic mechanisms. During evolution our ancestors lived in hot dry environments and subsisted primarily on herbivorous diets that were bereft of ionic sodium (salt). We therefore evolved on a diet which provided a sodium intake of <0.25g/day, and with the persistent threat of insufficiency. The availability of water is also of importance since episodes of dehydration require loss of salt to maintain osmolarity (McKinley et al., 1983). Subsequent rehydration can therefore not be completed without salt replacement (De Luca Jr et al., 2010; Weisinger et al., 1985). Therefore episodes of dehydration result in a greater salt requirement. Addition of salt to food only began about 5000 years ago when the Chinese discovered its preservative properties (He et al., 2010). Now the average salt intake in most countries around the world is approximately 9-12 g/day, with many Asian countries having mean intakes over 12g/day (Brown et al., 2009). It is therefore now rare to have inadequate salt intake, but the response to salt depletion may be seen experimentally in most mammals. In such models, depletion of salt leads not only to rapid physiological adjustments to maintain homeostasis, but also to profound behavioural changes in order to replenish body salt. Salt appetite is the physiological response to salt depletion and drives acquisition in compliment to conservation of salt.

1.2.1 Human model of salt appetite

Physiological salt appetite may be seen in humans in certain disease entities. In 1940, a case report by Wilkins and Richter (Wilkins and Richter, 1940) described the fatal case of a child with a renal salt losing condition due to adrenal insufficiency and hence low synthesis of aldosterone. The boy had an intense salt appetite. He unfortunately died when admitted to hospital for investigation which prevented him from raiding the kitchen cabinets for salt. Subsequent literature contains case series of disease states triggering a high salt intake (Kochli et al., 2005; Spiro et al., 1970). Although these case studies have been interpreted to show that humans exhibit salt appetite upon depletion, there have been no randomised controlled studies to confirm this or to clearly characterise the appetite in humans. There are a few healthy human studies either looking at the physiological effects of salt depletion (McCance, 1936) or salt taste whilst depleted (Beauchamp et al., 1990), neither of these studies have a control group. Only one cross over study has recorded salt intake from a shaker during depletion compared to the replete state and both subjects increased their intake (de Wardener and Herxheimer, 1957). Present research shows two aspects of salt appetite which are specific to humans:

1. Humans rarely ingest pure sodium chloride (Leshem, 2009)
2. Humans only ingest sodium as NaCl (Leshem, 2009) whilst rats will ingest alternative sodium salts (Nachman, 1962a).

1.2.2 Animal Models of salt appetite

Compared to human studies the literature regarding other species is more extensive. There are a variety of ways of inducing a salt appetite (discussed in detail in Chapter 2) and a variety of laboratory animals have been used. The resultant appetite has some consistent components. There is an increased intake of salt, both at the concentration which is normally drunk equally to plain water (Richter, 1936), and also at higher concentrations, which are normally avoided (Richter, 1936). There is motivation for high concentration salt. Animals will therefore work to access the high concentration, for example bar pressing (McCutcheon and Levy, 1972). The palatability of high concentration salt increases. This can be shown by stereotyped consummatory responses to the high concentration salt which change from those associated with aversive tastes, to those seen upon intra-oral infusion of palatable tastes (Berridge et al., 1984). In non-human models the appetite is specific for sodium not sodium chloride (salt) (Denton et al., 1988; Nachman, 1962b).

1.3 Physiological response to salt depletion

Prior to the expression of a salt appetite there is an initial physiological response to salt depletion to maintain haemodynamic stability. Total body salt is directly proportional to the volume of extracellular fluid (ECF) (Geerling and Loewy, 2008). ECF expansion increases kidney perfusion pressure resulting in increased salt excretion (Guyton, 1991). Salt depletion leads to a reduction in total body extracellular fluid. A reduction in ECF leads to a fall in blood pressure, which is detected by baroreceptors triggering an autonomic response, and a reduction in blood flow through the kidneys. The autonomic response and reduction in blood flow through the kidney both result in release of renin by the kidney. Renin enzymatically splits angiotensin from a blood protein called renin substrate (angiotensinogen) (Guyton, 1991). The activation of the renin-angiotensin-aldosterone system (RAAS) drives the main physiological response to salt depletion and results in conservation of salt by the kidneys. There are also adaptations by the gut and skin to reduce salt loss. Gut hormones, ghrelin and the guanylin peptide family, also play a role in sodium homeostasis, (Kemp et al., 2013; Mueller and Dieplinger, 2012), with effects on gut sodium absorption and natriuresis (loss of sodium from the kidney) although their response to salt depletion is unknown.

1.4 Peripheral stimulation of salt appetite

Whilst this physiological response maintains haemodynamic stability and prevents further salt loss, the salt need must be transmitted to the brain in order for an appetite to be generated. A number of peripheral signals have been investigated to ascertain the role that they play in stimulating salt appetite. They include plasma sodium concentration, aldosterone, angiotensin II, baroreceptors and a number of neuromodulators. Plasma sodium concentration intuitively seems an ideal signal but, whilst hypernatraemia, detected centrally by Na_x channels, inhibits ingestion of salt (Hiyama et al.) hyponatraemia does not stimulate salt appetite (Geerling and Loewy, 2008; McCance, 1936). This is understandable since the physiological importance of maintaining plasma sodium levels within a narrow range, and the avoidance of hyponatraemia means that a salt appetite needs to be triggered before hyponatraemia occurs.

The RAAS is activated in response to salt depletion and drives the physiological response to conserve salt. It is therefore ideally situated to also initiate salt appetite. Research confirms that aldosterone is a significant hormone in the stimulation of salt appetite. Due to the dual effects of aldosterone on

the kidney to retain salt, and also to stimulate appetite, both loss of aldosterone (Richter, 1936), by adrenalectomy creating a salt losing state, and administration (Fregly and Waters, 1966) cause salt appetite. The appetite stimulated is specific for salt and does not stimulate any other ingestion behaviour, such as drinking (Geerling and Loewy, 2008). Aldosterone release is stimulated by angiotensin II and glucocorticoids, but it is also released in the context of salt deprivation independent of these hormones (Okubo et al., 1997). A study on patients with aldosterone producing adenoma pre and post removal indicates that endogenous aldosterone production in humans also elicits a salt appetite (Pimenta et al., 2011). This study used urinary sodium as a marker of salt intake which has limitations (Lerchl et al., 2015). Animals depleted of aldosterone (via adrenalectomy) may express salt appetite, therefore aldosterone is clearly not vital for salt appetite. Also, supra physiological doses of aldosterone are required to induce salt appetite in rats (Wolf, 1964). Aldosterone crosses the blood brain barrier but in small amounts.

Angiotensin II (AII) is generated by the enzymatic cleavage of angiotensinogen to angiotensin I (AI) which is then processed to AII by angiotensin converting enzyme (ACE) (Geerling and Loewy, 2008). As previously mentioned, AII stimulates release of Aldosterone from the zona glomerulosa in the adrenal cortex and therefore has an indirect effect on salt appetite. There is also a direct effect, although in contrast to aldosterone, AII stimulates both water and salt intake, the latter being a delayed effect and maintained in the absence of aldosterone (Avrith and Fitzsimons, 1980). Both of these effects occur largely through stimulation of the AII type 1 (AT(1)) receptor but are dependent on the activation of different intracellular signalling cascades (Daniels et al., 2005). Salt appetite, due to furosemide induced salt depletion, in mice is inhibited by AT(1) antagonist (Crews and Rowland, 2005). Interestingly, administration of captopril and enalapril (ACE inhibitors) peripherally increases saline ingestion in rats (Minsker et al., 1984). Unlike losartan (AT(1) inhibitor), captopril and enalapril do not cross the blood brain barrier. The effect of ACE inhibition is therefore thought to be due to a reduction in peripheral conversion of AI to AII and therefore increased delivery of AI to the brain RAAS (Geerling and Loewy, 2008). The increasing recognition of a brain RAAS system (Hurley et al., 2014) questions the critical role of circulating AII. It is difficult to stimulate or increase salt appetite with peripherally administered AII (Geerling and Loewy, 2008), and the majority of experiments use centrally administered AII which create supra physiological concentrations and different patterns of neuronal activity compared to peripheral administration (McKinley et al., 1995). More physiological doses of both aldosterone (SC) and angiotensin (IC), which do not produce salt appetite alone, when co-administered produce salt appetite in both rats and baboons (Shade et al., 2002), suggesting synergy between these hormones (Geerling and Loewy, 2008). There also appears to be feedback

inhibition of AII induced salt appetite. This is revealed by an enhanced response to AII when various neurotransmitter antagonists are co-administered (Geerling and Loewy, 2008). The predominant feedback inhibition comes from oxytocin (Blackburn et al., 1992).

Neural inputs also play a role in activating salt appetite. Baroreceptors are stretch receptors located in vessel walls and hence detect blood pressure. This information is relayed, via afferent autonomic nerves, to the brain. Salt depletion leads to hypovolaemia but normal physiological response normally prevents a significant effect on arterial blood pressure and may lead to an increase (Webb et al., 1987). If blood pressure is maintained, then the potential role of baroreceptors to detect a salt need is limited. This is supported by the fact that destruction of the area of the brainstem innervated by these nerves has no effect on salt appetite (Schreihofer et al., 1999). Although blood pressure is maintained during hypovolaemia, postural hypotension occurs which would be detected by baroreceptors. Their possible role is supported by a study showing that salt appetite is reduced following transection of the autonomic nerves that transmit information from arterial baroreceptors (Thunhorst et al., 1994).

As previously mentioned, salt appetite is attenuated by the central administration of a number of peripherally and centrally produced neuromodulators. For example oxytocin, natriuretic peptide (released by the right atrium cardiomyocytes in response to stretch) (Blackburn et al., 1995), gastric peptide cholecystokinin (CCK) (Menani and Johnson, 1998) and vagally mediated signals (Curtis and Stricker, 1997), and neuromedin B (Flynn, 1996). This has given rise to the hypothesis that salt appetite is triggered by the same mechanism as thirst (mainly AII) with the additional removal of a dominant inhibitory signal that normally prevents salt appetite (Grafe et al., 2014; Stricker and Verbalis, 1987). In summary, it is likely that salt appetite is triggered by a multitude of peripheral signals, hormonal and neural, stimulatory and inhibitory which are then integrated centrally to regulate salt intake.

1.5 Neural circuits involved in initiating salt seeking behaviour

Once the brain has received the salt need signal, in order to control salt seeking behaviour, it must also receive information regarding the location of salt, via taste, and finally post-ingestive signals to terminate the appetite. Due to the properties of the blood brain barrier (BBB), which isolates the brain from the peripheral circulation, there are limited portals of entry for peripheral sensory

information to access neural circuits. Therefore the neural circuits implicated in the generation of salt appetite, by research to date, tend to stem from the nucleus of the solitary tract (NTS) (Titze et al., 2015) which receives direct neural input from systemic visceral receptors, areas of the brain where the BBB is incomplete, allowing neuronal access to those peripheral hormones which are unable to cross the BBB, and physiological conditions such as serum osmolarity and sodium concentration. These sensory portals with incomplete BBB are the circumventricular organs which include the subfornical organ (SFO), organum vasculosum of the lamina terminalis (OVLT), and the area postrema (AP). The information is then relayed to the forebrain to be integrated and the motivation to ingest salt relayed to the motor pattern generators (brainstem reticular formation) in order for salt seeking behaviour to occur (Geerling and Loewy, 2008).

The hypothesised networks involved in salt seeking behaviour have then been delineated by using complementary experimental techniques. Analysis of receptor expression, by immunohistochemistry or in situ hybridisation, identifies neurons capable of detecting hormones that convey a salt need. Many of the hormones involved in activating a salt appetite play a role in other processes, for example, AII is central to the thirst drive as well as salt appetite. Therefore receptor expression is not sufficient to confirm a role in salt appetite. Further evidence may be gained by showing activation during salt appetite and inactivation by subsequent salt ingestion, or activation by the peripheral mediators of salt appetite, mainly AII and aldosterone, administered centrally or peripherally. These experiments use *c-fos*, a depolarisation marker and hence an indication of neuronal activity, to identify areas and specific cell types. The essential nature of a brain region or cell type within the process may then be tested by lesion experiments and assessment of the impact on salt intake. Physical destruction may be used to target an area but is indiscriminate in complex areas containing different cell types and nuclei. Cell-specific inactivation is achievable by using inducible and non-inducible genetic methodology, or receptor expression guided destruction. Pharmacological studies, with systemic or precise IC administration, further identify regions, cell types and intracellular pathways involved in salt seeking behaviour and the impact of these pathways on salt ingestion. Networks involve the flow of information from one area/cell type to another. Mapping the sequential role of regions known to be involved and identifying new regions of interest has been achieved by tracing experiments. This involves a combination of retrograde and anterograde tracers to locate where neurons, identified as being involved in salt appetite, project to and receive inputs from. Additionally *in vivo* electrophysiology and FSCV experiments have also been very informative.

Use of the above techniques has identified regions that are involved in the flow and integration of the peripheral signals indicating a salt need. The main areas are the circumventricular organs, NTS, pre-locus coeruleus, and inner division of the external lateral parabrachial nucleus which then relay to forebrain sites, including ventrolateral bed nucleus of the stria terminalis (BSTvl), central nucleus of the amygdala, ventral pallidum, lateral hypothalamic area and ventral tegmental area.

In both rats and mice, circumventricular organs are activated during salt appetite, with furosemide induced salt depletion in mice stimulating *c-fos* expression in the SFO, medial preoptic nucleus (MnPO), AP, OVLT and SON (the latter male only) (Lu et al., 2012; Lu et al., 2009; Na et al., 2007; Rowland et al., 1996; Rowland et al., 2003). AT1 receptors are highly expressed in the circumventricular organs (Allen et al., 2000) and losartan blocks the expression of *c-fos* in the SFO and MnPO in response to salt depletion (Crews and Rowland, 2005). These experiments suggest that it is AII binding to AT1 receptors that leads to activation during salt depletion. Activation does not equate directly with behavioural function and lesions of the SFO, OVLT and surrounding hypothalamic tissue only cause a partial and variable decrease in the stimulation of salt intake by prolonged salt deficiency. In fact, despite exerting stimulatory and inhibitory effect on salt intake, no lesional studies have identified any of the AII-sensitive circumventricular organs as essential for the stimulation of salt appetite (Geerling and Loewy, 2008).

Mineralocorticoid receptors are expressed fairly extensively in the brain but will only act as an aldosterone receptor if there is co-expression of the enzyme 11β hydroxysteroid dehydrogenase type 2 to inactivate endogenous glucocorticoids. Lack of 11β hydroxysteroid dehydrogenase results in apparent mineralocorticoid excess (AME) due to unregulated activation of the MR by glucocorticoids. AME may be congenital or acquired due to excessive liquorice consumption or age related deficiency of 11β hydroxysteroid dehydrogenase. Neurons expressing both MR and 11β hydroxysteroid dehydrogenase are termed HSD2 neurons (Menani et al., 2014). These are found in the NTS and become *c-fos* positive following chronic and acute salt depletion. This is then rapidly corrected following ingestion of saline (Geerling et al., 2006). Conditional deletion of 11β hydroxysteroid dehydrogenase in the NTS results in salt appetite with mice showing a preference for saline compared to water up to a concentration of 3% (0.5M NaCl) (Evans et al., 2016). The effect of this deletion on salt intake following salt depletion was not tested. A case report has previously noted a compulsively high salt intake in a patient with mildly impaired 11β hydroxysteroid dehydrogenase (Ingram et al., 1996). The role of HSD2 NTS cells in salt appetite is not merely as an aldosterone sensor because *c-fos* expression is still seen in the absence of aldosterone. This

activation is thought to be due to their innervation from other areas implicated in salt appetite: dorsomedial NTS, central nucleus of the amygdala, paraventricular hypothalamic nucleus and the AP (the latter possibly exerting a tonic inhibition) (Geerling et al., 2006; Geerling and Loewy, 2008).

HSD2 cells of the NTS project mainly to the BSTvl, the pre-locus coeruleus (pre-LC) and the inner division of the external lateral parabrachial nucleus (PBel inner) (Geerling and Loewy, 2006). They also send minor axonal projections to the midbrain VTA, lateral and paraventricular hypothalamic nuclei, central nucleus of the amygdala, and periaqueductal grey matter (Geerling and Loewy, 2006). This suggests that these sites contribute to the development of salt appetite.

The ventrolateral bed nucleus of the stria terminalis (BSTvl) is implicated by neuroanatomical and physiological experiments as a key site in the regulation of salt appetite. Lesion experiments substantially reduce salt appetite (Reilly et al., 1994), although they are too imprecise to differentiate between the various BNST sub-nuclei. Also, tracing experiments reveal HSD2 inputs to the BSTvl as well as inputs from AII sensitive neurons in the SFO and OVLT, and A2 noradrenergic neurons from the NTS (Geerling and Loewy, 2008). It is highly interconnected with the central nucleus of the amygdala (Dong et al., 2001) and also receives inputs from the hypothalamus including a dense projection from the appetite-stimulatory peptidergic neurons in the arcuate nucleus of the hypothalamus (Shin et al., 2008). The BSTvl sends out massive projections to the lateral hypothalamic area, paraventricular hypothalamic nucleus (Geerling and Loewy, 2008) and other regions including the VTA (Jennings et al., 2013). Neurons in the brainstem reticular formation are considered the gatekeeper for controlling ingestive behaviour; the BST sends both direct and indirect projections to these regions. The central amygdala (CeA) also sends axonal projections to these regions and in turn receives inputs from many of the other key areas involved in salt appetite.

Another area that has been consistently implicated in salt appetite is the pre-locus coeruleus (pre-LC) and inner division of the external lateral parabrachial nucleus (PBelinner) which contain cells that become *c-fos* positive during salt appetite and may be identified by their expression of the transcription factor Forkhead box protein 2 (FoxP2). FoxP2+ cells in the pre-LC project to sites including the ventral pallidum (VP), hypothalamic nuclei and the VTA. PBelinner cells project to the paraventricular (PVH) and dorsomedial (DMH) hypothalamic nuclei with weaker connections to the LHA and VTA (Shin et al., 2011).

The projection to the VP is interesting since the VP has been identified as a hedonic hotspot, mediating “liking” reactions to sensory pleasures, and as such plays an important role in the “liking”

of food rewards (Castro et al., 2015). As well as tracing experiments, its role in salt appetite has been suggested by *in vivo* electrophysiological recordings which show a change in the firing properties of its neurons, elicited by the taste of hypertonic saline, according to the physiological salt need of the rat (Tindell et al., 2006). This study showed a doubling in the amplitude of VP neural firing peaks in response to hypertonic saline when the animal was salt deplete compared to the salt replete state. In comparison VP neural activity to palatable sucrose was always high (Tindell et al., 2006). These results suggest a role for this area in encoding a change in the reward value of hypertonic saline in response to salt depletion.

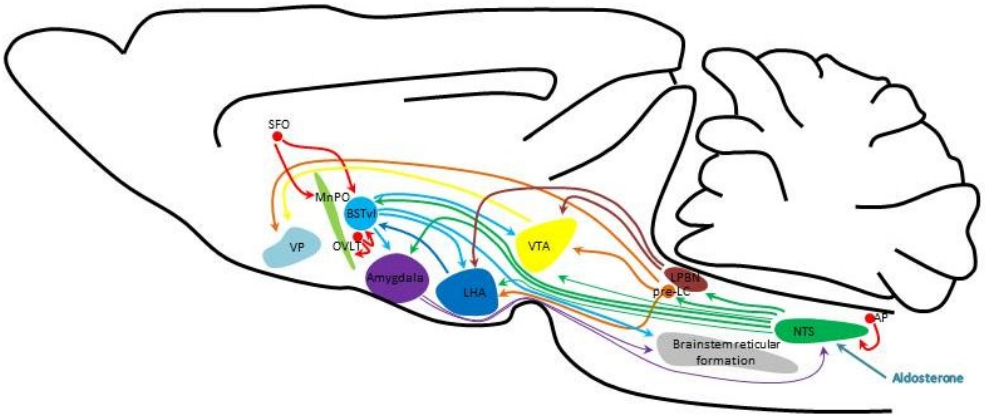

Figure 1.1: Neural pathways involved in salt appetite

Schematic diagram of a mouse brain in sagittal section showing the major regions and pathways that have been identified as involved in salt appetite. SFO: subfornical organ; MnPO: medial preoptic nucleus; VP: ventral pallidum; BSTvl: ventrolateral bed nucleus of the stria terminalis; OVLT: organum vasculosum of the lamina terminalis; LHA: lateral hypothalamus; VTA: ventral tegmental area; LPBN lateral parabrachial nucleus; pre-LC: pre-locus coeruleus; NTS: nucleus of the solitary tract; AP: area postrema.

The VP is part of the mesocorticolimbic circuitry, receiving mesolimbic dopamine projections from the VTA. The VTA also receives inputs from the pre-LC and PBelinner and is implicated in salt appetite. I will first explore the midbrain dopamine neurons and their role in behaviour before focusing on the contribution the system makes to salt seeking behaviour.

1.6 Midbrain dopamine neurons

Midbrain dopamine neurons are located in three nuclei, the VTA (A10), the retrorubral field (RRF, A8) and substantial nigra pars compacta (SNc, A9). They are also a few scattered in the substantia nigra pars reticulate (SNp). The VTA is sub-divided into the parabrachial pigmented (PBP) and the paranigral (PN) nuclei (Swanson, 1982). The caudal linear nucleus (CLi), interfascicular (IF), and rostral linear nucleus of the raphe (RLi) may also be considered VTA sub-regions (Oades and Halliday, 1987). Dopamine neurons are characterised by long axonal projections which terminate in dense and complex arbours (Matsuda et al., 2009) with the vast majority of dopamine neurons sending projections to non-overlapping areas (Swanson, 1982). The projection targets are spread widely throughout the brain (Figure 1.2). The classical classification of separate nigrostriatal, mesolimbic and mesocortical pathways is an oversimplification. It has been shown that SN neurons project to the cortical and limbic areas in addition to the striatum, whilst cells within the VTA project to both striatal, limbic and cortical areas (Björklund and Dunnett, 2007). The limbic projection targets include the nucleus accumbens, central nucleus of the amygdala and the caudal striatum. As well as the caudate putamen, other basal ganglia structures are also innervated by midbrain dopamine neurons, including segments of the globus pallidus, parts of the ventral pallidum and the subthalamic nucleus (Björklund and Dunnett, 2007). Recent literature has started to identify subgroups within the VTA dopamine neurons with regard to their location, projection targets, different electrophysiological properties and response to aversive or appetitive stimuli (Björklund and Dunnett, 2007; Brischoux et al., 2009; Matsumoto and Hikosaka, 2009; Matsumoto and Takada, 2013)

The widespread innervation of different regions is reflected in the range of important day to day functions that the midbrain dopamine neurons have been shown to influence including fine motor control (Cousins and Salamone, 1996; Nieoullon and Amalric, 2002), learning and memory (Grace et al., 2007; Matsumoto and Takada; Wise, 2004), reward-related and motivational behaviour (Salamone and Correa; Schultz, 1998; Wise, 2004).

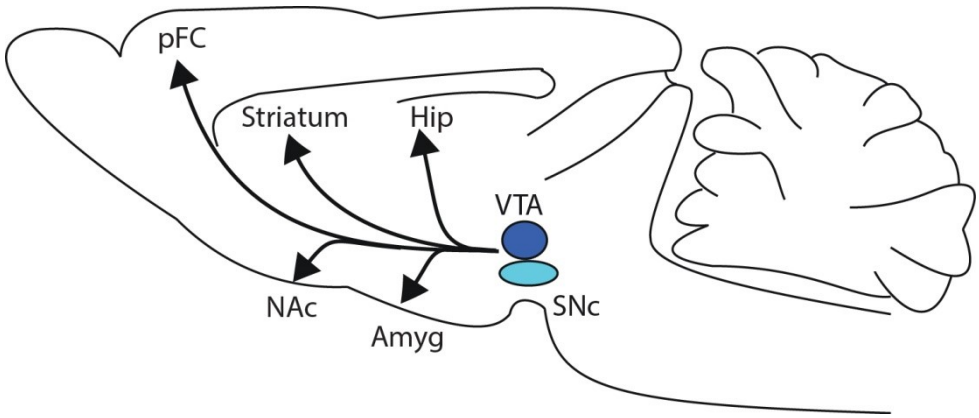

Figure 1.2: Location and projection targets of midbrain dopamine neurons

Schematic diagram of a mouse brain in sagittal section showing the projection targets of midbrain dopamine neurons. The cell bodies of the most widely studied subsets of dopamine neurons are located in the midbrain regions of substantia nigra pars compacta (SNc) and the ventral tegmental area (VTA). Neurons from the SNc and VTA send out long axonal projections to a number of different regions of the brain, some of which are illustrated in this figure, thereby having a significant post-synaptic influence. pFC: pre-frontal cortex; Hip: hippocampus; NAc: nucleus accumbens; Amyg: amygdala.

Electrophysiological studies show that dopamine neurons display two principal types of spontaneous firing patterns *in vivo* – single spike firing and burst firing. Single spike firing is characterised by single action potentials fired at irregular (or regular) intervals while burst (phasic) firing is composed of two to ten spikes fired in close succession interspersed with irregular single spikes (Grace and Bunney, 1984a, b). Single unit recordings, from putative dopamine neurons in primates, show that during an operant task, dopamine neurons are phasically excited by unexpected food rewards (Schultz, 1997). Supported by subsequent extensive studies this has led to the hypothesis that dopamine neurons' phasic activity encodes reward prediction errors, the discrepancy between an expected reward and the actual outcome (Lammel et al., 2014). Thus the omission of a reward had the opposite effect and negative reward prediction errors were associated with silent periods (Schultz, 1997). This hypothesis has been confirmed in humans using fMRI (D'Ardenne et al., 2008) and in mice with optogenetically single-cell identification of VTA dopamine neurons (Cohen et al., 2012). This is not the only function of dopamine neurons and it has been shown that dopamine neurons are excited by

aversive stimuli (Brischoux et al., 2009; Lammel et al., 2012; Matsumoto and Hikosaka, 2009) as well as inhibited by aversive stimuli (Brischoux et al., 2009; Ungless et al., 2004).

A reward prediction error is not an experience of pleasure. Initial experiments involving self-stimulation indicated that the dopamine synapse was the area in the brain where a sensory input was translated into a hedonic message and therefore experienced as pleasure, euphoria or "yumminess" (Wise, 1980). Recent research has shown the mesolimbic dopamine system not to be as hedonic as previously described (Berridge and Kringelbach, 2015). It's role lies more in "wanting" which drives motivation for a reward (Berridge, 2009).

A dysfunction in dopamine neurons has been linked to several disorders. For example, drugs of abuse, despite their diverse chemical substances and initial binding protein, converge on the VTA and NAc with common acute functional effects (Nestler, 2005). Chronic exposure to drugs of abuse also causes common chronic functional changes in the VTA-NAc pathway. Consistent with this is the observation that certain drugs of abuse, under particular experimental conditions, can result in cross tolerance and cross-sensitisation to one another with respect to locomotor and rewarding effects (Nestler, 2005). Schizophrenia, in its acute psychotic state, is associated with an increase in dopamine synthesis, dopamine release and resting-state synaptic dopamine concentrations (van Os and Kapur, 2009). This is reflected by the fact that all current pharmacological treatments for schizophrenia block dopamine receptors. In chronic schizophrenia negative symptoms are linked with reduced ventral striatal dopamine activity (Radua et al., 2015). Dopamine neurons also play central role in the pathogenesis of Parkinson's Disease in which there is loss of dopaminergic neurons in the SNc resulting in akinesia, rigidity, and tremor at rest (Oertel and Schulz, 2016). Since midbrain dopamine neurons have been shown to be central to motivation and wanting, this would suggest an important role in appetite where there is wanting and motivation for food.

1.7 Role of dopamine neurons in general appetite

The literature regarding the role of the mesolimbic system in general appetite is much more extensive than that on salt appetite. There are likely to be similarities between neural pathways involved in general appetite and salt appetite. I will therefore start to summarise the literature pertaining a role for the mesolimbic system in general appetite before moving on to the role of the dopamine neurons in salt appetite.

Animals eat for different reasons. These may be broadly categorised as homeostatic, whereby circulating signals inform the brain of available energy stores and in response the brain controls food intake (Morton et al., 2014); non-homeostatic when the determinant for food intake is palatability and pleasantness not need (Berthoud, 2006); and emergency when certain conditions which are immediately life threatening, for example hypoglycaemia, trigger feeding in deviance of the homeostatic control. The neural pathways involved in each are likely to have many overlaps but there will also be differences. During a meal the contribution to intake by these control circuits also varies. It is thought that a meal/eating bout is initiated generally by non-homeostatic mechanisms and ended after ingestion of the desired amount (often controlled by homeostatic factors) (Alonso-Alonso et al., 2015). This interaction and the fact that satiety is one of the major determinants controlling the palatability of food leads to some debate as to the presence of a truly independent non-homeostatic drive (Morton et al., 2014). It is important to keep in mind these different controls when interpreting the literature and be aware of the physiological state of the animals. In many of the experiments the animals are fasted or chronically calorie restricted in order to generate a hunger, or water deprived to generate a thirst, and therefore drive the observed behaviour. The findings may not then be applicable to other physiological states.

Dopamine deficient mice are hypoactive and profoundly hypophagic, lacking life sustaining feeding behaviour. Within a few minutes of L-dihydroxyphenylalanine (L-DOPA) administration the $DA^{-/-}$ mice become more active and consume more food than controls (Zhou and Palmiter, 1995). This indicates that dopamine is essential for feeding. Research assessing dopamine release in the NAc further supports the role of the mesolimbic system in appetite. Dopamine levels are elevated in the NAc following ingestion of food (Hernandez and Hoebel, 1988) and, in the context of sham feeding, there appears to be a sucrose concentration dependent response (Hajnal et al., 2004). The magnitude of dopamine release is dependent on the physiological state – hunger v satiety (Wilson et al., 1995). Whilst these experiments relied on microdialysis to measure levels of dopamine, more recent experiments use *in vivo* fast scan cyclic voltammetry (FSCV) which is able to measure dopamine "transients" and hence dopamine released by phasic firing (Owesson-White et al., 2012). These show that, in rats, unpredicted food reward and reward predictive cues evoke a phasic increase in dopamine in the striatum. This release is subregion specific and depends on experience, hence in rats, with limited experience, release was confined to the NAc -core, whilst in rats, trained on a discriminative stimulus paradigm, release was in the NAc-core and dorsomedial striatum (Brown et al., 2011). *In vivo* FSCV NAc measurements have also shown that food restriction increases the magnitude of dopamine spikes evoked by food (Cone et al., 2014). Ghrelin, a peptide released by the

stomach in association with hunger, also led to an increase in the magnitude of dopamine spikes in response to sugar pellet delivery but only when infused directly into the LH and not the VTA. The potentiation of dopamine spikes by lateral ventricular ghrelin was blocked by intra-VTA orexin receptor antagonist (Cone et al., 2014). The role of the mesolimbic system in appetite is also supported by imaging studies which show an increase in striatal activity in humans corresponding to the pleasantness of food (Small et al., 2003). Showing release of dopamine in response to food reward, does not necessarily attribute the role of dopamine in the "enjoyment" of food. Looking at facial response of animals and human babies the "liking" or "disgust" in response to a taste may be measured (Berridge, 2012). Using this technique it is shown that hyperdopaminergic mutant mice have higher "wanting" but not "liking" for sweet reward (Peciña et al., 2003).

As shown by the classic Pavlovian experiments, food reward is anticipated following a learning process. Dopamine plays a role in this as shown by dopamine levels in the nucleus accumbens in response to cues for food and sweets. Taste may be used as a cue (conditioned stimulus, CS) when the associated nutritive gastric load is controlled by an intragastric cannula. A taste CS leads to an increase in dopamine in the NAc (Mark et al., 1994). In monkeys visual cues, encoding the probability and size of a liquid sucrose reward, stimulate dopamine neurons, measured by extracellular recordings, in proportion to size and probability of the reward (Tobler et al., 2005). This is not simple coding for reward value since, whilst unpredicted rewards lead to stimulation in proportion to the size of the reward, a predicted reward did not lead to stimulation. This illustrates the reward prediction error concept of dopamine neurons in the context of appetite.

If food reward leads to increase NAc dopamine, does increased dopamine lead to increased intake? Intra-accumbens amphetamine microinjections (which leads to dopamine release) enhances the ability of a Pavlovian reward cue to trigger lever pressing for a sucrose reward in rats (Wyvell and Berridge, 2000) but this result is contrary to human experience in which amphetamines have been used as an appetite suppressant (Weintraub et al., 1984). Amphetamines have a broad based reuptake inhibitor action, affecting serotonin, adrenaline and noradrenaline as well as dopamine. Dopamine deficient ($DA^{-/-}$) mice show some ingestive behaviour which is resistant to the hypophagic effects of moderate dose amphetamine but following restoration of dopamine signalling in the caudate putamen, by viral gene therapy, $DA^{-/-}$ mice manifest normal amphetamine induced hypophagia. Mice deficient in noradrenaline, adrenaline, dopamine D_2 receptors, dopamine D_1 receptors, serotonin 2C receptors and neuropeptide Y do not show blunting of the hypophagic effect of moderate dose amphetamine. This study suggests that the action of amphetamines to reduce

appetite is due to the dysregulation of striatal dopamine signalling (Cannon et al., 2004). $DA^{-/-}$ mice hypophagia is not merely due to the lack of activation of dopamine receptors since dopamine receptor agonists fail to promote sustained feeding in $DA^{-/-}$ mice (Kim and Palmiter, 2003). This would suggest that it is the dynamics of dopaminergic signalling which is critical (Cannon et al., 2004) or that the level is critical, hence activation of behaviour is dependent on an inverted U-shaped function of dopamine activity. In the case of appetite, optimal levels of dopamine activity result in food-seeking behaviour (Roitman et al., 2004). However, too little dopamine, exemplified in $DA^{-/-}$ mice, inhibits feeding. Too much dopamine, as due to amphetamine also results in inhibition of intake during appetite. Dopamine may have different effects in different target regions, and in the same region at different intensities (Berridge et al., 2010).

The mesolimbic system is adaptive, with previous experience influencing subsequent behaviour(Johnson and Kenny, 2010). Most drugs of abuse lead to a release of dopamine in the NAc. Chronic drug use leads to neuroadaptation in the reward circuits prompting an escalation of intake. Imaging experiments illustrate this adaptation by showing changes in the availability of D2 receptors and other changes in the NAc in monkeys following chronic cocaine self-administration (Nader et al., 2006). D2 receptor expression has been shown to play a similar role in compulsive-like food seeking in rats (Johnson and Kenny, 2010).

Analogous to my discussion on the peripheral stimulation of sodium appetite, general appetite is also stimulated by peripheral signals. Two of the main peptides involved are leptin, which is produced primarily by adipocytes and signals satiety, and ghrelin which is produced by the gut and is elevated by food restriction and fasting (Cone et al., 2014; Gualillo et al., 2003; Murakami et al., 2002). Ghrelin crosses the blood brain barrier and ghrelin receptors are expressed throughout the brain including the VTA, arcuate nucleus of the hypothalamus and lateral hypothalamus (LH). Central administration of ghrelin stimulated increased dopamine levels in the NAc (Jerlhag et al., 2006). It also increases dopamine release due to food reward (Cone et al., 2014) and food predictive cues (Cone et al., 2015). This is due to its effect on LH neurons projecting to the VTA and not a direct effect on the VTA dopamine neurons (Cone et al., 2014). Imaging studies of patients with congenital leptin deficiency show the opposite effect of leptin. Leptin replacement therapy attenuates striatal activity elicited by visual images of food (Farooqi et al., 2007). Therefore the role of dopamine in general appetite is supported by the known effect of the two main hormones, involved in controlling homeostatic appetite, modulates its release in the NAc (Castro et al., 2015). The importance of dopamine in general appetite is further indicated by research examining the effect of nutritional

state (for example blood glucose (Bello and Hajnal, 2006) and other metabolic hormones on dopamine. For example insulin (Jones et al., 2016), MCH (Pissios et al.), CCK (Frommelt et al., 2013), orexin (Choi et al., 2012), GLP-1 (Anderberg et al., 2014) and amylin (Mietlicki-Baase et al., 2015).

In addition to the homeostatic control of appetite, the aim of which is to maintain stable body fat stores over time, there is an emerging concept that neuro-circuits exist that are normally inhibited, but when activated in response to emergent or stressful stimuli they can override the homeostatic control of energy balance (Morton et al., 2014). One such stimulus is hypoglycaemia which results in inadequate delivery of glucose to the brain (neuroglucopenia or glucoprivation). The neurocircuits activated by this stimulus, cause neuroendocrine and autonomic responses which raise blood glucose. In addition there is a potent and sustained increase in food intake (neuroglucopenic or glucoprivic feeding) which overrides controls exerted by the energy homeostasis system (Morton et al., 2014). There have been no studies assessing the role of the mesocorticolimbic circuity in driving this hyperphagia, although anecdotally patients report enhanced enjoyment of food when hypoglycaemic which would suggest a role.

In summary, dopamine neurons play an essential role in appetite/feeding behaviour and it is likely that this role is dependent on either optimal levels of dopamine or a specific dynamic pattern of dopaminergic signalling.

1.8 Role of dopamine neurons in salt appetite

Similar to general appetite, motivation and enjoyment are key elements of salt appetite. Whilst satiety reduces the motivation and enjoyment of hedonist foods like sucrose, they never become aversive (Berridge, 2012). Salt appetite is unique in that it involves motivation and enjoyment of hypertonic saline, with suppression of its innate aversiveness. The ability to experimentally switch the response to hypertonic saline, from aversive to appetitive, means that salt appetite has been used as a model to explore the role of the mesolimbic system in reward (Cone et al., 2016; Robinson and Berridge, 2013). However our understanding of the role of dopamine neurons in salt appetite is limited.

The most direct evidence quoted is Hoebel *et al.* (1989) who recorded a 200% increase in extracellular dopamine using microdialysis in the NAc of salt depleted rats during the ingestion of saline. Unfortunately his work was only published in abstract form and therefore cannot be fully

evaluated. The results are supported by a recent study which used FSCV to measure NAc dopamine transients in response to hypertonic saline in order to investigate how physiological state shapes dopamine signals evoked by outcomes and their predictors (Cone et al., 2016). Intra-oral 0.45M NaCl evoked phasic dopamine release in the NAc only in salt depleted mice and not mice that were salt replete, salt depleted but then sated, nor salt deplete but unable to detect salt due to topical amiloride administration (Cone et al., 2016). *C-fos* studies show an increase in activity in VTA neurons when a CS for hypertonic saline, for which the CS-UCS trials occurred during the replete state, was presented during salt depletion. There was no increase in *c-fos* in the salt depleted state alone, nor following expression of salt appetite without the CS (Robinson and Berridge, 2013). ERK-dependent activation of *c-fos* reflects a summation of activity-dependent calcium influx over seconds to minutes (Cruz et al., 2015). Therefore the *c-fos* promotor is only induced in consistently and strongly activated neurons. Hence an absence of increase in *c-fos* during salt appetite without a CS may reflect a difference in the pattern and intensity of dopamine neuron activity, compared to when a CS is present, rather than an absence of dopamine neuron activity. Salt appetite induced by intracerebroventricular AII appears to result in a different effect on dopamine neurons, with an increase in *c-fos* in VTA neurons following administration of AII but prior to expression of the salt appetite (Grafe and Flanagan-Cato, 2016). Pharmacological studies also support a role of dopamine neurons in salt appetite with a reduction in intake following administration of a dopamine receptor (D2) antagonist in the context of sham drinking (Roitman et al., 1997), where a gastric fistula prevents absorption and therefore satiation of salt appetite. D1(5) and D2(3) antagonists also reduce intake in standard drinking (Liedtke et al., 2011).

Indirect evidence for the involvement of the midbrain dopamine neurons comes from studies looking at the effect of salt appetite on areas that these neurons project to. In sham drinking, activation of the NAc is shown by staining for the immediate early gene *c-fos* (Voorhies and Bernstein, 2006). *In vivo* electrophysiological recordings from the NAc, during intraoral infusions of 0.45M NaCl solution, show that neurons in the NAc shell responded in a mixed manner in the replete state but when depleted there is a switch from predominantly excitations to predominantly inhibitions. In the core, overall activity was altered only after salt depletion was corrected (Loriaux et al., 2011). The reduced activity in the NAc shell, in response to hypertonic saline, during salt depletion support the theory that decreases in activity to rewarding stimuli may promote positive hedonic reactivity (Loriaux et al., 2011). Another area of interest is the ventral pallidum (VP), which receives dopaminergic inputs from the VTA and GABAergic inputs from the NAc (Smith et al., 2003). *In vivo* recordings show an increase in firing in response to oral 1.5M NaCl, following salt depletion.

The firing pattern triggered by hypertonic saline following depletion mirrors the pattern recorded in response to the hedonic taste of sucrose (Tindell et al., 2006).

Amphetamine has a direct effect on dopamine release and there are similarities and interactions between amphetamine and salt appetite. Induction of salt appetite produces dendritic morphology changes in the nucleus accumbens (Roitman et al., 2002) analogous to that seen with amphetamine. There is also reciprocal cross-sensitisation between amphetamine and salt appetite (Clark and Bernstein, 2004), and pre and post-natal dietary salt manipulation alters adult offspring salt intake (Contreras, 1993) and sensitises them to amphetamine (McBride et al., 2008). This implies that the same pathways are involved in salt appetite and the effects of amphetamine and hence implicates the role of dopamine in salt appetite.

1.9 Salt detection and gustatory pathways

Although the reinforcing properties of taste are attenuated if the nutritional value of the food is dissociated from taste (Beeler et al., 2012), highly palatable food will motivate consumption even in a calorically replete state. Taste and the detection of salt are therefore essential for salt seeking behaviour. There are five basic taste qualities, sweet, sour, salt, bitter and unami. The first two are appetitive whilst the latter two are aversive. Salt is unique in its ability to generate two distinct behavioural responses dependent on concentration. In the normal state low concentration (<100mM) is appetitive whilst high concentration is aversive. Low concentration salt is detected by a dedicated population of taste-receptor cells (TRCs) which express epithelial sodium channel E and mediate the behavioural attractions to low concentration NaCl (Chandrashekar et al., 2010). The E channel is blocked by amiloride (Doolin and Gilbertson, 1996). Whilst it was previously thought that the five basic taste qualities were detected by dedicated populations of TRCs, it has recently been shown that high concentration salt (>300mM l) recruits the two primary aversive taste pathways by activating the sour and bitter taste sensing cells (Oka et al., 2013). Although this is slightly at odds with studies that have shown an inability to express salt appetite when amiloride is administered (Robinson and Berridge, 2013). Information from the taste buds is conveyed via the peripheral gustatory nerves and then mainly the chorda tympani branch of cranial nerves VII to the rostral one third of the lateral NTS (Geerling and Loewy, 2008). Information is then relayed to the forebrain either directly or indirectly depending on the species.

1.10 Hypothesis and aims

Based on the studies that show the VTA receives inputs from areas known to be important in triggering salt appetite(Shin et al., 2011), VTA dopamine neurons are activated when there is a salt appetite (Grafe and Flanagan-Cato, 2016; Robinson and Berridge, 2013), dopamine is released in response to ingestion of hypertonic saline in the salt depleted state (Cone et al., 2016), and D2 antagonists attenuate intake (Roitman et al., 1997) I hypothesised that dopamine neuron stimulation would increase salt intake. The ability to modulate patients' motivation to ingest salt would be clinically useful and may help to mitigate the detrimental effects of excessive salt intake. Therefore extending the understanding of the role of this system in salt intake is important.

The aim of this thesis is to explore the effect of modulating VTA dopamine neurons during salt seeking behaviour and hence test this hypothesis. In order to achieve this, I established a reliable model by which salt seeking behaviour may be characterised (Chapter 2). I then obtained *in vivo* optogenetic control of VTA dopamine to enable me to modulate VTA dopamine neuron activity during salt seeking behaviour and ascertain the effect, reported in Chapter 3. I then went on to complement my optogenetic experiments by using an alternative technique to control neuronal activity, chemogenetics, reported in Chapter 4. Finally, in chapter 5, I report my results by investigating the effect of optogenetic modulation of dopamine neuron on sucrose intake after an overnight fast and need free salt concentration preference.

Chapter 2 Salt appetite: induction and characterisation

2.1. Introduction

Prior to investigating the role of the dopamine neurons in salt seeking behaviour I established a reliable assay of salt appetite in the laboratory. This required a robust method of inducing a salt appetite followed by an assay which described the appetite not merely by intake of hypertonic saline with reference to water but by the change in preferred salt concentration.

Salt appetite may be induced in many mammals but the majority of historical papers used rats for experimental models (Fregly and Waters, 1966; RICE and RICHTER, 1943; Richter, 1936). With the advent of genetic engineering, and the relative ease of manipulating the genome of mice compared to rats, the use of mice has increased. My wish to use optogenetic technology to target dopamine neuronal activity, necessitated a rodent dopamine neuron cre line. TH Cre rats are available (Witten et al., 2011) but the behaviour testing facilities available to me were for mice. I therefore used a mouse model of salt appetite. There are size and physiological differences between rats and mice therefore not all models of salt appetite may be used in mice (Johnson et al., 2015). Hence the first section of my experimental plan was to identify an appropriate model of salt appetite and establish it in our laboratory.

A variety of models to induce salt appetite have been used in the literature. These models either induce the physiological conditions that lead to the generation of an appetite or manipulate the hormones and peptides involved in signalling a salt need. The former includes salt depletion or removal of extracellular fluid whilst the latter includes manipulation of the aldosterone/mineralocorticoid or RAAS systems. Models also vary as to whether they induce pure salt appetite or also trigger thirst.

The initial animal experiments reflected the clinical presentation of salt appetite due to adrenal insufficiency. Therefore the first animal model of salt appetite was adrenalectomy (Richter, 1936). It was subsequently discovered that the opposite hormonal manipulation, high dose mineralocorticoids, also stimulated saline ingestion in mammals (RICE and RICHTER, 1943). When aldosterone was purified, it was shown to stimulate salt intake (Wolf, 1964). Aldosterone requires regular dosing of supraphysiological levels over a number of days (Geerling and Loewy, 2008) so the mineralocorticoid (MC) receptor (MR) agonist deoxycorticosterone acetate (DOCA) is more often

used. This provokes a robust sodium appetite in rats (Morris et al., 2010). In mice, adrenalectomy fails to provoke a salt appetite and results with DOCA are variable (Johnson et al., 2015). Although the angiotensin model works in mice, it requires central administration and pre-treatment with aldosterone or DOCA (Weisinger et al., 1996). I planned to use optogenetics, which requires surgery. I therefore chose not to use a salt appetite protocol that also involved surgery and hence avoided central drug administration.

Depletion of body salt by prolonged low salt diet induces salt appetite in rats. Some behavioural changes are present by 2 days (Garcia et al., 2008) but consistent appetite shown by a 2 bottle test takes 8 days (Stricker et al., 1991), this appetite is blunted by oestrogen so is less in female rats (Stricker et al., 1991). In mice low salt diet alone is not sufficient to drive a salt appetite (Rowland et al., 2004) (Denton et al., 1988) (although in the latter the experiment was only for 2 days), and chronic low salt diet does not induce an increase in saline intake (Rowland and Fregly, 1988). Dehydration results in loss of salt, via a naturesis, which is postulated to be a mechanism to maintain plasma osmolarity (McKinley et al., 1983). Therefore on rehydration a salt appetite is generated (De Luca Jr et al., 2010; Weisinger et al., 1985). This method has not been tested in mice. Other methods that sequester extracellular fluid also stimulate salt appetite, for example peritoneal dialysis with a hyperoncotic colloid, which draws extracellular fluid into the peritoneal space (Toth et al., 1987), or a subcutaneous injection of polyethylene glycol leading to sequestration at the injection site (Stricker, 1981). The most reliable method of depleting a mouse of salt, sufficient enough to generate a salt appetite, has been via the use of the loop diuretic furosemide. Furosemide, acting in the kidney, binds to the Cl^- binding site of the $Na^+2Cl^-K^+$ carrier, thereby inhibiting the luminal uptake of these ions in the thick ascending Loop of Henle and hence reabsorption of salt from urine (Davison, 2005). Furosemide treatment, in the context of a low salt diet, generates a salt appetite (Jalowiec, 1974). This model has been tested in mice (Denton et al., 1988) and used extensively (Liedtke et al., 2011; Rowland et al., 2004; Rowland et al., 2003). It is therefore this last method that I decided to use.

The main site of furosemide action is the kidney but at high dose there are extra renal effects. In humans high dose may result in ototoxicity, but this is normally in the context of renal impairment and intravenous administration (British Medical Association and Royal Pharmaceutical Society of Great Britain). Furosemide is also a subtype-selective $GABA_A$ receptor antagonist (Korpi and Lüddens, 1997). $GABA_A$ receptors are expressed on many neuronal types, including dopamine neurons, and when furosemide is administered intra VTA this results in opiate motivation change

(Ting-A-Kee et al., 2013). The half-life of furosemide in humans is less than 2 hours and in rats is 50 minutes at a dose of 40mg/kg which, based on surface area, equates to a human dose of 320mg (Hammarlund and Paalzow, 1982). If behavioural testing is performed 24 hours after dosing there should be no furosemide present minimalizing the risk of a direct effect of furosemide on dopamine neurons. The furosemide model of salt depletion has been used in many studies assessing the mesolimbic system (Liedtke et al., 2011; Tindell et al., 2006). Therefore whilst furosemide induced acute salt depletion is an acceptable model for my experiments, it is prudent to avoid high dosing. Liedtke et al (2011) used 1.2mg ip (approximately 52 mg/kg) once daily for two days, this was my starting point. The dose used should ideally salt deplete the mouse sufficient to induce a salt appetite without long term consequences nor drug toxicity.

My initial experiments were carried out to establish the acceptance of the low salt diet; to assess the reliability of drinking tubes, and ensure the tolerability of repeated acute furosemide induced salt depletion using an established protocol.

2.2. Methodology

Animals

Mice were housed in cages of 3-4 mice, kept at a constant temperature and maintained on a 12-hour light/dark cycle. Prior to any changes in diet, all mice were fed on standard rodent chow and water *ad libitum*. Since I had initially planned an *ex vivo* neurophysiology component to my experiment, my initial experiment was set up using TH GFP transgenic mice [B6.Cg-Tg(TH-GFP)21-31 ⁄ C57B6] (Matsushita et al., 2002), which express fluorescent green protein in dopamine neurons, from our group's colony. For subsequent non-optogenetic experiments male C57BL/6 mice were obtained from Charles River. Animal husbandry and experimental procedures were performed in full compliance with the United Kingdom Animal (Scientific Procedures) Act of 1986 Amendment Regulations 2012. PPL 70/7438.

Tolerability of low sodium diet

<table>
<tr><th></th><th>number</th><th>Feed</th><th>Water</th><th>Saline</th></tr>
<tr><td>chow</td><td>3</td><td>RM1 (0.25% Na)</td><td rowspan="3">distilled water via standard drinking bottle and sipper</td><td rowspan="2">none</td></tr>
<tr><td>Na Deplete</td><td>4</td><td rowspan="2">RM<0.025% Na, Special Diets Services</td></tr>
<tr><td>Low Na diet</td><td>4</td><td>0.075M via lixit valve</td></tr>
</table>

The above groups were established. After 7 days of habituation to the above set up the mice were placed in metabolic cages (Tecniplast, Buguggiate, Italy) for 24hrs in order to collect urine and calculate urinary sodium excretion (prior to any treatment). Weights were recorded over 4 weeks.

Establishing salt depletion protocol

Twenty four C57BL/6 mice age 3 – 4 weeks were housed in cages of four mice and established on low salt feed (RM<0.025%Na, Special Diets Services) with access to 0.075M NaCl solution and distilled water via volumetric drinking tubes. The tubes were constructed of a lixit valve attached to a bung inserted into a 15ml falcon tube. A hole was drilled in the tip of the tube to enable flow of fluid through the valve and filling of the tube. These tubes did not leak so could be used long term without the risk of the mice being without fluid. The mice were weighed daily to monitor tolerability of the set up. Day 6 the mice were singly housed. After a 24hr habituation period, 24hr food, water and 0.075M NaCl intake was recorded. The mice were then re-grouped. On day 15, 3 weeks of weekly salt depletion studies were started. Each study consisted of daily intraperitoneal (ip) injections with cage changes and weighing for 2 days, then a two bottle test on day 3. Between studies the set up reverted to baseline. The mice were assigned to one of three groups, all mice in one cage received the same treatment. The groups were: saline control group, which received ip 0.9% saline injection (Animalcare Ltd); furosemide control group which received ip furosemide (Hameln Pharmaceuticals) 26.1mg/kg injections; and the treatment group which received ip furosemide 26.1mg/kg injections. Both control groups had access to 0.075M saline whilst for the treatment group this was removed. For the two bottle testing, individual mice were placed in clean cages lined with absorbent paper and covered with a metal grill lid. After 15min habituation 2 drinking tubes were offered through the bars one containing distilled water the other 0.3M NaCl. These tubes were constructed in accordance with the Monell Mouse Taste Phenotyping Project (Tordoff and Bachmanov, 2003) with the exception that a ball bearing sipper was used to reduce the

incidence of leakage. Intake was measured by weighing the tubes at baseline and after 15, 30 and 60min.

Statistical analysis

Data are presented as mean ± standard error. Statistical comparisons (Graphpad Prism 6, La Jolla, CA) were made by using 2-way analysis of variance (ANOVA) with repeated measures. Where results refer to an ANOVA analysis for either main effect or the effect of genotype, the F value is given. In all other instances, the p value refers to the Tukey's or Sidak's comparison. Longitudinal analyses were kindly performed by Anuskha Fernando.

2.3. Results

Tolerability of low salt feed

Only 6 incomplete urine samples were collected. This was partly due to my inexperience and the failure of urine to flow into the collecting vessel of the metabolic cages. Such small numbers meant statistical analysis was not possible. However, urinary sodium concentrations (Fig. 2.1a) show that the feed control group (which had normal chow) were not conserving sodium indicating that salt intake was higher than physiological need. That rodent chow contains higher salt than needed for rats, has previously been shown (Martus et al., 2005). My results suggest that the same is true for mice. The salt control group and salt deplete group were conserving salt. (The lowest NaCl concentration detectable by the machine is 20mmol). The low urinary sodium of the salt control group indicates that the NaCl intake of this group matched physiological need and there was not excessive intake of the available 0.075M NaCl. Or it may be that it takes effort to obtain fluid from a lixit valve compared to a free flowing water bottle with a standard sipper, therefore intake of saline was reduced and thus this group were relatively salt deplete. When I switched the type of sipper used for the water to match that used for 0.075M NaCl, then the intake of 0.075M NaCl was significantly increased (Fig. 2.1b).

Acute salt depletion

Liedke et al (2011) used a furosemide dose of 1.2mg ip (approximately 52 mg/kg) (Liedtke et al., 2011). When I used this dose for repeat depletions the furosemide group's weight gain was reduced

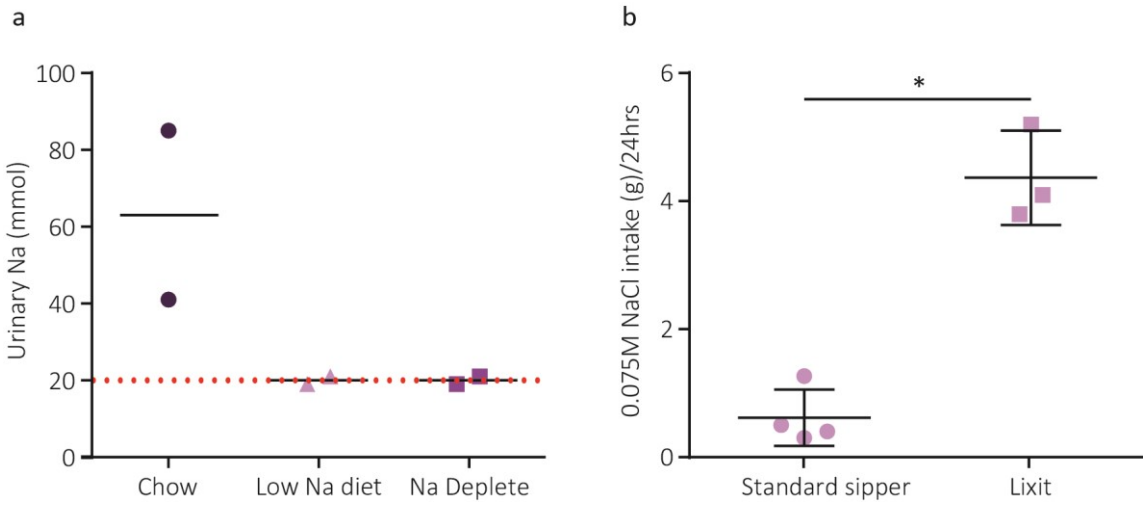

Figure 2.1: Mice sodium balance during chronic salt depletion

a. Urinary sodium concentrations of mice with access to standard chow; low sodium feed with 0.075M NaCl available via a lixit valve drinking tube; low sodium chow without access to NaCl (water was available to all groups via a water bottle with standard sipper). **b.** Intake of 0.075M NaCl from a lixit valve drinking tube with accompanying water available from a standard sipper or a lixit drinking tube ($p<0.05$).

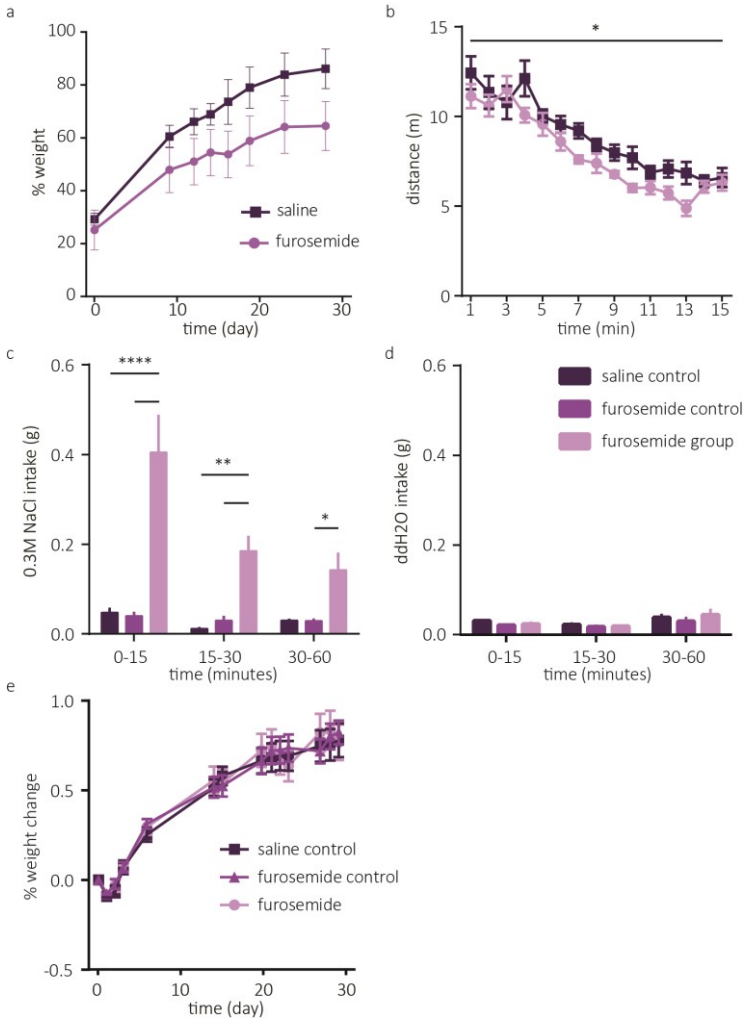

Figure 2.2: Salt appetite stimulated by acute furosemide driven salt depletion

a. Weight gain during repeat 1.2mg (mean 57 mg/kg) furosemide driven sodium depletion (n=8) and control (n=7) (time x treatment $F(7, 91)=3.674$ $p<0.005$ pairwise comparisons ns). **b.** Locomotor activity following repeat 1.2mg furosemide driven salt depletion (treatment $F(1, 13)=5.177$ $p<0.05$). **c.** 0.3M NaCl intake during 2 bottle testing following 26.1mg/kg furosemide driven salt depletion (n=8) and controls (n=8) (treatment $F(2, 21)=56.19$ $p<0.0001$, time x treatment $F(4, 42)=4.551$ $p<0.005$ pairwise comparison furosemide group vs controls $p<0.0001$ 1-15 min, $p<0.005$ 15-30 min). **d.** ddH_2O intake during 2 bottle testing following 26.1mg/kg furosemide driven salt depletion (n=8) and controls (n=8) (treatment $F(2, 21)=0.7$ n.s. time x treatment $F(4, 42)=0.4382$ n.s.). **e.** Weight gain during repeat 26.1mg/kg furosemide driven salt depletion (n=8) and controls (n=8) (treatment $F(2, 21)=0.0361$ n.s. time x treatment $F(26, 273)=0.5634$ n.s.)

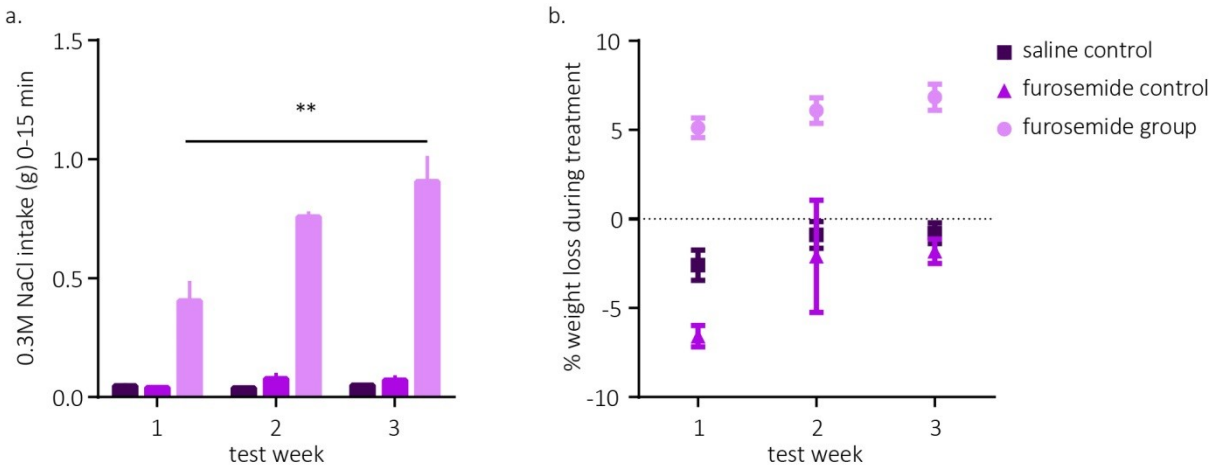

Figure 2.3: Sensitisation of sodium appetite following repeated sodium depletion

a. 0.3M NaCl intake during 0-15 minutes of 2 bottle testing (treatment $F(2, 21)=173.6$ $p<0.0001$, week $F(2, 42)=11.21$ $p<0.0005$, treatment x week $F(4, 42)=9.184$ $p<0.001$ pairwise comparisons furosemide group week 1 vs week 2 and week 1 vs week 3 $p<0.001$, week 2 vs week 3 n.s.). b. weight loss from day 1 of treatment to day 3 prior to 2 bottle test.

(Fig. 2.2a), this was of concern. I therefore tested them in the open field for a measurement of general wellbeing. They were significantly less active when assessed in open field testing one week after the third 2 bottle test (Fig.2.2b). Since this suggested that the furosemide treatment had significant long term side effects in addition to the desired effect of salt depletion, the dose was reduced to 26.1mg/kg. The natriuresis induced by this dose was sufficient to produce a significant salt appetite. This was shown by a 2 bottle test of 0.3M NaCl and ddH_2O (Fig. 2.2 c & d). Intake of 0.3M NaCl was significantly greater than controls and in comparison to ddH_2O. Using this dose of furosemide there was no difference in weight gain over time between the groups.

In rats, repeated salt depletion causes a sensitisation like effect where salt appetite increases with each dose (Johnson and Thunhorst, 1997). However, not all mouse studies have shown this (Denton et al., 1988; Rowland and Fregly, 1988). My results show sensitisation with increased intake of 0.3M NaCl in the first 15 minutes with subsequent episodes of salt appetite (Fig. 2.3a). The weight loss on treatment (a marker of extracellular fluid loss) was constant over the 3 weeks (Fig. 2.3b) indicating that the increase in 0.3M NaCl intake was not due to a greater renal response to repeated doses of furosemide and therefore a more profound salt depletion.

The established method of testing for salt appetite is via a 2 bottle test comparing intake of plain water with hypertonic saline that is aversive in the normal state (Lucas et al., 2007). Whilst running the above studies I became more aware of the limitations of this method. Water bottles also tend to leak especially when being moved in order to weigh them, this results in an inaccurate intake measurement and frequent exclusions of mice due to lost data necessitating larger cohort sizes than would be required if these technical issues were addressed. Whilst lixit valves reduce leakage, ease of consumption is reduced and some mice fail to master the use of lixit valves. In addition, most human salt ingestion is from food, it is difficult to produce food with no salt, the choice is therefore between food with different concentrations of salt, not zero salt. The traditional 2 bottle test fails to reflect this. Human studies have looked at preference for different salt containing foods (Leshem and Rudoy, 1997) and preference for salted food has recently been tested in rats (McKinley, 2013) in the context of salt appetite. A salt gel canteen has previously been used with reliable results (Rowland et al., 2004). I therefore established a jelly canteen assay and I then went on to validate the jelly canteen assay for measuring sodium appetite.

2.4. Method

Canteen salt jelly testing of salt appetite

0.4% agar salt jelly was made by boiling distilled water with agar and the appropriate weight of sodium chloride to make 0M, 0.075M, 0.15M, 0.3M or 0.9M according to the experiment. 0.075M 0.4% agar jelly was available in the baseline setup with low salt feed, in 200ml glass tubs, but switched to a plain 0.4% agar jelly for salt depleted mice when injected with furosemide. 30mm glass petri dishes were used to hold the test jellies. These were labelled with a marker pen beneath. For later experiments they were scented by wiping on a tissue wet with almond oil to aid habituation. Intake was measured by recording the weights every 10-12 minutes.

2.5. Results

In the first 10 minutes, salt depleted mice had an intake of high concentration salt jelly (0.3M and 0.5M) which was significantly greater than plain jelly. This difference was maintained at 10 – 20 minutes for the 0.3M NaCl jelly. After 20 minutes the appetite had dissipated with no significant difference between the concentrations. There was no significant difference between intake of 0.3M and 0.5M NaCl jelly at any time interval (Fig. 2.4a). Intake of 0.3M NaCl jelly revealed a significant salt appetite in the furosemide treated mice compared to the control group. This appetite was maintained for 20 minutes (Fig. 2.4b).

Pilot studies and the available literature informed a canteen test using concentrations of NaCl of 0.075M, 0.15M and 0.3M. This canteen was used to compare different doses of furosemide (Fig. 2.5). Weight loss during treatment (Fig. 2.5a) showed an overall significance of treatment (one-way ANOVA), this was due to a significant difference between control and the two furosemide groups. There was no significant difference between the furosemide groups suggesting that the amount of natriuresis plateaus. Comparison of salt jelly intake in the first 12 minutes for all 3 groups showed that both furosemide doses produced a salt appetite with a significant higher intake of 0.3M NaCl than saline control (Fig. 2.5b). Examination of the pattern of jelly consumption within each furosemide group (Fig. 2.5c and d) showed that 10mg/kg produced the most ideal pattern with a significant difference between intake of all concentrations in the first 12 minutes. The group treated with 26.1mg/kg did not differentiate between 0.15M and 0.3M NaCl jelly.

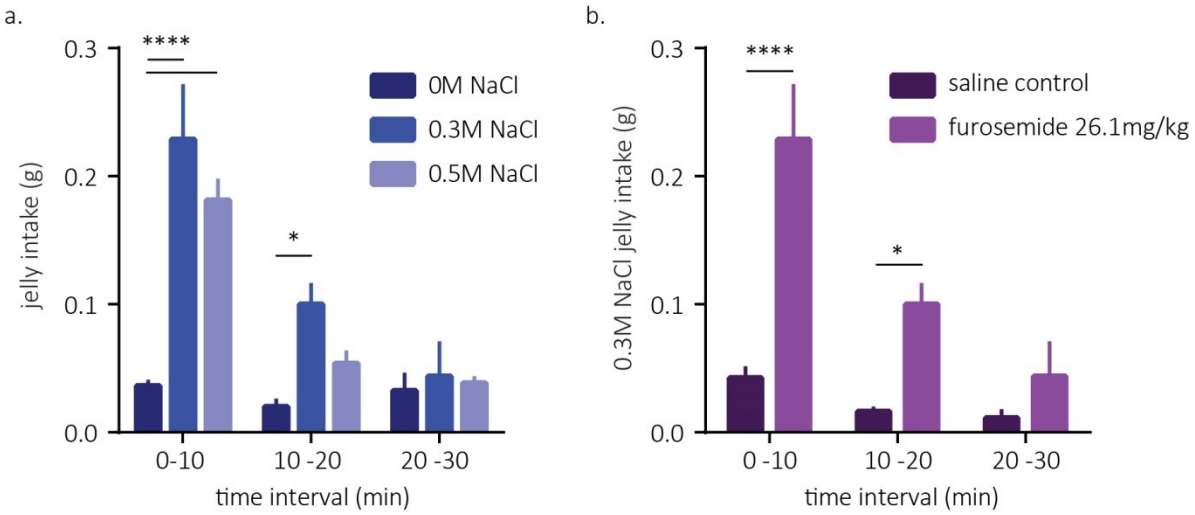

Figure 2.4: Agar jellies are as robust at measuring a salt appetite as a 2 bottle test

a. Jelly intake of salt depleted mice (n=8) (26.1mg/kg furosemide) (time x concentration $F(4, 63)=6.006$ $p<0.0005$, pairwise comparisons 0-10 min 0M vs 0.3M and 0M vs 0.5M $p<0.0001$, 10-20 min 0M vs 0.3M $p<0.05$). **b.** 0.3M salt jelly intake of salt replete (saline control) (n=8) and salt deplete (furosemide group) mice (time x treatment $F(2, 42)=6.039$) $p=0.005$, pairwise comparisons 0-10min furosemide group vs control $p<0.0001$, 10- 20 min furosemide group vs control $p<0.05$)

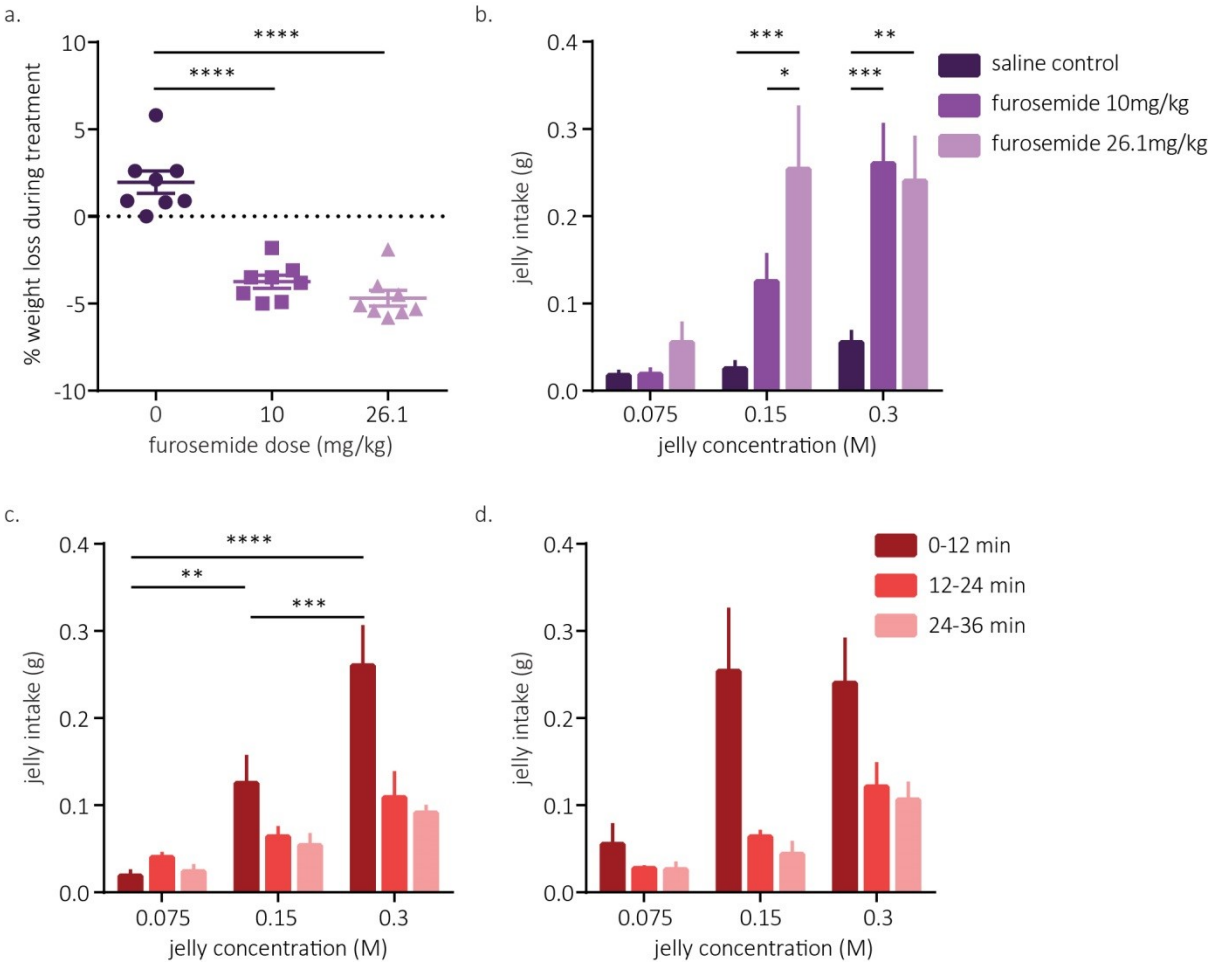

Figure 2.5: Comparison of furosemide dose

a. Weight loss during treatment (treatment F(2, 21)=51.94 p<0.0001, pairwise comparison saline vs 10mg/kg and saline vs 26.1mg/kg p<0.0001, 10mg/kg vs 26.1mg/kg n.s.). **b.** Comparison of salt jelly intake 0-12 minutes between groups treated with 0, 10mg/kg or 26.1mg/kg furosemide once daily for two days (concentration x treatment F(4, 42)=2.747 p<0.05, pairwise comparisons 0.15M NaCl jelly saline vs 26.1mg/kg p<0.001, 10mg/kg vs 26.1mg/kg p<0.05; 0.3M NaCl jelly saline vs 10mg/kg p<0.001, saline vs 26.1mg/kg p<0.005). **c.** 10mg/kg furosemide group jelly intake over time (time x concentration F(2, 42)=4.445 p<0.005 pairwise comparisons 0-12 minutes 0.075M vs 0.15M p<0.005, 0.075M vs 0.3M p<0.0001, 0.15M vs 0.3M p<0.001). **d.** 26.1mg/kg furosemide group jelly intake over time (time x concentration F(4, 42)=2.41 n.s. concentration F(2, 21)=8.247 p<0.005, time F(2, 42)=13.55 p<0.0001).

2.6. Discussion

In this chapter I have optimised the dose of furosemide required to obtain salt depletion in mice significant enough to drive a salt appetite. Whilst higher doses of furosemide have been used in previous studies, my results show that higher doses do not produce an improved appetite and incur the risk of adverse effects. A jelly canteen test is a reliable way to test salt appetite and using concentrations of 0.075M, 0.15M and 0.3M produced a clear effect of concentration on intake. This is therefore an effective assay to assess the impact of interventions on salt appetite. This range of concentrations also reflects the transition of salt detection from amiloride sensitive (appetitive) to amiloride insensitive (aversive) pathways. The aversive, amiloride insensitive pathway, detects salt at concentrations of 0.15M and above(Chandrashekar et al., 2010). 0.3M salt is detected by both pathways but is avoided by C57BL/6 mice (Tordoff et al., 2014). Repeated episodes of salt depletion resulted in enhanced salt appetite (sensitisation) which is of interest in isolation but also means that a cross over study design is not appropriate for interventional experiments. My data are unable to explain if this is true sensitisation or due to increased familiarity with the test setup. To test this, the setup could to be changed between testing or one could look for cross sensitisation with the locomotive effect of amphetamines.

All of these experiments involved naïve mice which had not had a previous intervention, for example surgery. They were handled only briefly 6 times prior to testing and had 15 minutes habituation to the test cage before receiving the jellies. As shown by my results, there was good consumption of the jellies. My later experiments contrast markedly. Mice subjected to more stressful protocols, involving surgery and tethering, required more habituation and handling otherwise many mice showed no ingestion in the first ten minutes. A higher dose of furosemide was also required to generate a reliable appetite.

In the following chapters I use the protocols reported in this chapter to test the effect of modulating dopamine neuron activity on salt appetite.

Chapter 3 Optogenetic excitation and inhibition of dopamine neurons and the effect on sodium appetite

3.1 Introduction

Having established a reliable way of inducing and measuring salt appetite in the previous chapter I then wished to modulate the activity of VTA dopamine neurons during the salt jelly canteen test in order to explore their role in salt seeking behaviour. Optogenetics is an ideal tool to do this.

Optogenetics is the use of light and genetics to manipulate and monitor the activities of defined cell populations (Song and Knopfel, 2016). It involves the introduction of a microbial opsin gene into a cell, most commonly neurons. The opsin is a light sensitive ion channel or pump. Light activation of the opsin opens the ion channel. Dependent on the selectivity of the channel this will either depolarise and activate the neuron, or hyperpolarise and inhibit the neuron. Therefore, expression of the opsin enables control of the neuron's activity with light *in vivo*, in mobile or anaesthetised animals, or *in vitro*. This technology was first described in mammalian neurons in 2005 (Boyden et al., 2005) using Channelrhodopsin 2, a naturally occurring algal protein. The kinetics of the channel are rapid with a ~10ms latency from illumination to spike peak (Boyden et al., 2005). Thus multiple pulses of light are able to drive trains of multiple spikes and hence replication of *in vivo* firing patterns with precise temporal control. Halorhodopsin (NpHR) is a chloride pump that is activated upon illumination with 580nm yellow light. This results in an increase in intracellular chloride, hyperpolarisation and therefore inhibition (Zhang et al., 2007). A great strength of optogenetics is that it may be targeted in a cell-type specific manner. One way of achieving this is Cre technology. Cre is an enzyme that catalyzes recombination between *lox*P sites (Sauer and Henderson, 1988). Depending on the orientation of the flanking *lox*P sites, Cre may be used to invert and excise (Fig. 3.1) (Schnutgen et al., 2003). The combination of a Cre recombinase–dependent opsin-expressing virus and a transgenic animal expressing Cre in a defined cell type enables optogenetic techniques to be cell-type specific (Tsai et al., 2009).

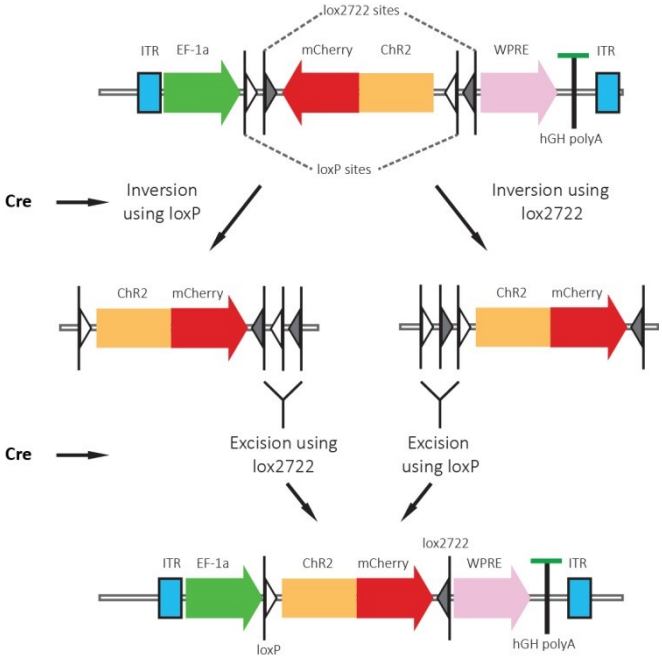

Figure 3.1: Schematic representation of the Cre dependent AAV

The ChR2/mCherry gene is flanked by two sets of incompatible lox sites. Injection into BAC DATiCre (described as DATCre from now on) mice results in inversion of the gene enabling transcription in dopamine neurons where Cre is expressed.

There are important limitations to this approach. Cell-specific expression is achieved by the expression being under the control of the promotor of a protein which is only expressed in that cell type. Cell specificity is therefore dependent on the cell specificity of that promotor. In the VTA dopamine neurons have traditionally been identified as expressing TH (Ungless and Grace), and accordingly TH-Cre knock-in mouse driver lines have been used in approximately 80% of the studies involved in Cre-dependent targeting of midbrain dopamine neurons e.g. (Adamantidis et al., 2011; Chaudhury et al., 2013; Tsai et al., 2009). However, a recent study has shown that expression under the control of the TH promotor exhibits non dopamine cell-specific expression within and around the VTA. Use of the dopamine transporter (DAT) promotor appears not to share this problem (Lammel et al., 2015). This illustrated the need to test for non cell-specific expression when using Cre technology. Using knock-in transgenic mice, where the transgene is inserted in place of the endogenous gene, also renders the mice heterozygous for the targeted gene. There is therefore a risk of altering expressing rates of the protein, TH or DAT, with a subsequent behaviour phenotype. A Bacterial Artificial Chromosome (BAC) transgenic mouse overcomes this particular concern.

However, this technology may also have its own confounds due to the increased gene dosage contained within the large BAC DNA sequences (Ting and Feng, 2014).

In addition to cell specificity, spatial precision can be achieved by stereotactic viral injection and further refined by the location of the optical fibre tip. Optogenetics therefore gives temporal, spatial and cell specific control of neuronal activity and may be used in mobile animals to assess the behavioural effect of neuron activity modulation.

Optogenetics in the mesolimbic dopamine system

The midbrain dopamine system is a complex system of dopamine neuron subtypes with different axonal projections and inputs and distinct anatomical, molecular and electrophysiological features (Björklund and Dunnett, 2007; Ikemoto, 2007; Margolis et al., 2008). There is also evidence that dopamine neurons release co-transmitters including GABA (Tritsch et al., 2012) and glutamate (El Mestikawy et al., 2011). The expression of tyrosine hydroxylase may be used to identify dopamine neurons by immunohistochemistry. It has been shown that expression may change over time and vary in response to changes in hormonal status and functional demand (Björklund and Dunnett, 2007) but since it is the most sensitive and consistent single marker available I have used it throughout my thesis. Optogenetics has offered a great opportunity to explore the complexity of the mesolimbic dopamine system in the context of behaviour (Adamantidis et al., 2011; Chaudhury et al., 2013; Tsai et al., 2009; Tye et al., 2013; Zhang et al., 2015). Of special interest to this thesis are the studies deciphering the role of the midbrain dopamine system in reinforcement and reward value. I will therefore describe the studies which have examined the role of dopamine neurons in passive and active reinforcement as well reinforcement in the context of food reward. One aspect that is of particular interest is the role of timing, of optogenetic modulation of dopamine neurons, on the reinforcing effect of dopamine neuron activity. Then I will discuss those studies which examine the role of dopamine in reward value.

Electrical intra-cranial self-stimulation studies originally led to the VTA being labelled as a “pleasure centre” (Wise, 1980). Although this is now generally disputed (Berridge and Kringelbach, 2015) the theory was based on the reinforcing effect on behaviour of VTA stimulation. Direct, phasic, optogenetic stimulation of VTA dopamine neurons results in release of dopamine in the NAc (Adamantidis et al., 2011) and this is able to drive passive reinforcement as shown by the generation of a conditioned place preference (Tsai et al., 2009). VTA dopamine neurons have been shown to respond to aversive stimuli and the absence of an expected reward as well as to an unexpected

reward. If distinct subsets of dopamine neurons are responsible for these responses then targeted optogenetic stimulation of VTA dopamine neurons would be able to elicit conditioned place aversion (CPA) as well as CPP. Whilst VTA dopamine neurons lack markers to distinguish them within the VTA, subsets may be targeted using optogenetics by stimulating the neurons projecting into the VTA from different regions. The two main inputs to the VTA are the laterodorsal tegmentum (LDT) and lateral habenula (LHb)(Lammel et al., 2012). Lammel *et al* used anatomical tracing studies to decipher the location of dopamine neurons in the VTA to which neurons in the LDT and LHb project and where these dopamine neurons project to. The results suggested that these dopamine subsets project to the NAc lateral shell and mPRC respectively. Injection of a rabies virus expressing ChR2 into the VTA resulted in retrograde ChR2 expression in cells projecting to the VTA. By implantation of the optic fibre over the LDT or LHb, optogenetics was used to interrogate the behavioural attributes of the dopamine neurons receiving inputs from these different regions. Stimulation of the LDT elicited a CPP whilst stimulation of the LHb elicited a CPA. This suggests that 2 different subsets of dopamine neurons are involved in the appetitive and aversive pathways and this is reflected in differing reinforcing properties, negative and positive.

Results from studies looking at active reinforcement, involving work to gain stimulation, is more mixed. One study, using optogenetic stimulation in mice, failed to replicate the electrical intra-cranial self-stimulation experiments, leading to the conclusion that phasic stimulation of dopaminergic neurons alone, in the absence of food reward, was not reinforcing and therefore was not effective in driving self-stimulation-like behaviour in mice (Adamantidis et al., 2011). On the other hand, in rats, using nose pokes rather than levers, the conclusion was that optical stimulation of dopamine neurons in the VTA was sufficient to support vigorous intracranial self-stimulation (Witten et al., 2011). In the latter study, during the first trial the ports were baited with food but thereon there was no association between the optical stimulation of dopamine neurons and food. Subsequently, using DATCre mice, self-stimulation of VTA dopamine neurons via lever press, in the absence of food reward, has been used to establish a model which closely resembles addiction (Pascoli et al., 2015).

In the context of food reward the present optogenetic studies support the role of dopamine in reinforcement. Adamantis *et al* combined *in vivo* optogenetic stimulation of dopaminergic neurons in the VTA during a food-seeking operant task (Adamantidis et al., 2011). Unilateral optogenetic targeting of the VTA in (Th)::IRES-Cre knock-in mice was performed. Using FSCV to measure dopamine transients in the NAc, optogenetic stimulation of the VTA with 5ms of light-pulse trains

delivered at 25Hz was shown to be most efficient at inducing dopamine release. *In vivo* optrode recordings revealed that efficiency of light-evoked action potentials decreased at frequencies greater than 25Hz. Stimulation was linked to pressing of the "active" lever, pressing either the active or inactive lever resulted in a food reward. The mice were food restricted during the duration of the training and testing session. ChR2 mice pressed the active lever significantly more than the controls suggesting the activation of dopaminergic neurons induced positive reinforcement and assigned a preference to the active lever but only in the context of food reward. When neither lever resulted in food reward there was no difference between the ChR2 mice and controls. Interesting, although the increased lever pressing activity resulted in more food rewards, food consumption between the two groups was the same.

Hamid *et al* stimulated dopamine neurons during two different stages of an adaptive decision making task that is closely related to the reinforcement learning framework (Hamid et al., 2016). The task started at "lights on" when a randomly chosen port lit up, indicating that the rat may obtain a reward. Upon placing its nose in the port (Centre-In) after a variable delay, an auditory "Go cue" sounded. This prompted the rat to make a movement to an adjacent side port, either left or right (Side-In). The rat needed to learn through trial and error which side port was currently more likely to elicit a sugar pellet reward. Left and right had separate reward probabilities and these probabilities changed periodically without any explicit signal. ChR2 was expressed bilaterally in VTA dopamine neurons of TH-Cre rats. The effect of stimulation, in a burst firing pattern (10ms pulses of blue light at 30Hz, 0.5s total duration), at the start of the task, "lights on", and at the time of the reward prediction error (RPE), "Side-in", was assessed. The effect on motivation, by measuring latency, and the effect on reinforcement, by its impact on subsequent "Side-in" choice, was assessed. Consistent with Adamantis *et al*, phasic dopamine neuron stimulation at the time of the RPE was reinforcing, and the same "Side-in" choice was more likely to be repeated on the next trial. But this was unrelated to food reward, since the effect was independent of the choice resulting in a food reward or not. Inhibition resulted in the opposite effect, when Halorhodopsin was used to inhibit dopamine neurons at "Side-in", the probability that the same left/right choice occurred on the next trial was reduced. The reinforcing effect was time dependent and only occurred following stimulation at "Side-in" and not "Lights-on". Stimulation at the time of "lights-on" had an effect on motivation but it was dependent on the rat's stage of engagement in the task when the stimulation occurred. If the rat was already engaged, waiting near the central port for a port to light up, the very short latencies became slightly longer whilst if the rat was not engaged there was a significant reduction in the latency. This indicates that mesolimbic dopamine is less important for the initiation of simple, cue-

evoked responses when a task is already underway, but is critical for motivating "flexible approach" behaviours (Hamid et al., 2016).

Domingos *et al* used optogenetic stimulation of VTA dopamine neurons to add reward value to a choice and then assessed how physiological state alters reward value. In two bottle testing sucrose is preferred to sucralose. Using Dat-Cre knock-in mice to unilaterally express ChR2 in VTA dopamine neurons, phasic stimulation was linked to licking of the sucralose sipper. This resulted in a switch in preference for the sucralose. Food restriction increased the value of sucrose relative to sucralose plus optogenetic stimulation whilst leptin reduced it (Domingos et al., 2011). Interestingly, Steinberg et al (Steinberg et al., 2013), who used optogenetic stimulation in rats to investigate the role of dopamine in cue-reward learning, in a supplementary experiment failed to show that manipulation of dopamine neuron activity during consumption of distinct but equally preferred sucrose solutions changed their value.

Whilst approximately 65% of neurons in the VTA are dopaminergic, approximately 30% are GABAergic and 2% to 3% are glutamatergic (Nair-Roberts et al., 2008). Optogenetics has offered the opportunity to further the understanding of the role of these neurons in reward processing. Optogenetic stimulation of VTA GABA neurons elicits a CPA (Tan et al., 2012) supporting previous *in vitro* studies that show that GABAergic neurons tonically inhibit VTA dopamine neurons (Johnson and North, 1992). The inhibition of VTA dopamine neurons by VTA GABA neurons *in vivo* was also shown by Cohen *et al* (2012). Previous *in vivo* electrophysiological studies aimed at distinguishing the precise role of VTA dopamine and GABA neurons in reward processing have been limited by the ability to establish the identity of the neuron recorded (Ungless and Grace, 2012). Cohen *et al* (2012) combined *in vivo* electrophysiological recordings with optogenetic "tagging" to distinguish between dopamine and GABA neuron activity during the association of different odour cues with appetitive and aversive outcomes. They identified three types of neurons within the VTA based on responses to odour and outcome. Type I (52%) showed phasic excitation after reward-predicting odours and a reward prediction error; the other half showed persistent activity during the delay between odour and outcome that was modulated positively (type II) or negatively (type III) by the value of outcomes. Optogenetic "tagging" identified type I as dopaminergic and type II as GABAergic neurons suggesting that VTA GABAergic neurons signal expected reward, a key variable for dopaminergic neurons to calculate reward prediction error (Cohen et al., 2012).

VTA GABA neurons also project to sites beyond the VTA, including the NAc and Substantia Nigra (van Zessen et al., 2012). Van Zessen *et al,* using VGat-ires-Cre mice to express ChR2 in VTA GABA

neurons unilaterally, examined the effect of GABA neuron stimulation, within the VTA or at the NAc axonal projections, on consumption during a cue-reward conditioned set up and free-reward consumption. The tip of the optic fibre was implanted over either the VTA or NAc. Optical activation of GABA neurons occurred either during the 5s cue or the first 5s following reward (sucrose) delivery. VTA GABA stimulation during the first 5s following reward delivery significantly reduced consummatory licking, which rebounded in the 5s after termination of VTA GABA stimulation (van Zessen et al., 2012). During free-reward consumption, VTA GABA stimulation also resulted in a decreased consumption during the time of optical activation. No effect on consumption was observed when optical activation occurred within the NAc. This suggests that it is the local VTA effect of GABA stimulation, notably dopamine neuron inhibition, which led to this effect rather than the release of GABA within NAc.

Experiments have also used optogenetic dopamine neuron stimulation to explore the contribution of the VTA to models of depression (Chaudhury et al., 2013; Tye et al., 2013). Whilst not directly related to the subject of this thesis they are important with regard to the principles and techniques of using optogenetics to interrogate VTA dopamine neurons.

Optogenetics offers a great opportunity but results may be difficult to interpret without careful consideration to the subtleties of physiological neuronal activity and non-linearities between neural activity and the studied behaviour (Kravitz and Bonci, 2013). These factors may account for some of the discrepancies in the experiments described above. Even when an attempt is made to record the effect of optogenetic dopamine neuron stimulation on dopamine release, there is disagreement between results. Adamantidis *et al* used fast-scan cyclic voltammetry (FSCV) to measure the release of dopamine in the NAc induced by optogenetic stimulation of dopamine neurons in the VTA. This showed that 5ms light-pulse trains delivered at 25Hz were most efficient at inducing dopamine release. However, a similar experiment in rats, suggested 50Hz is ideal (Bass et al., 2013). This illustrates why whilst the above papers were a great source of technical information to create a framework for establishing and using optogenetics, it was important to validate each stage throughout this thesis. As the optogenetic literature base grows then more consensus will hopefully develop.

The literature therefore confirms that optogenetics can be used to examine dopamine neuron function in the context of behaviour. It also shows that stimulation of dopamine neurons generally promotes reward related behaviour. Therefore in the context of salt appetite I would expect stimulation of dopamine neurons to result in an increase in salt intake.

In this chapter I present the experiments performed to initially test the transgenic mouse line to ensure there was no adverse phenotype, and evaluate expression of ChR2-mCherry in VTA dopamine neurons. I then go on to ensure that I am able to stimulate dopamine neurons and that this stimulation is able to influence behaviour. Finally, I explore the effect of optogenetic modulation of dopamine neurons on salt appetite.

3.2 Methods/experiments

Animals

For the optogenetic studies, male DATCre heterozygous mice and wild-type litter mates on a C57BL/6 background were used (Turiault et al., 2007). The original mice were kindly gifted by Prof. Francois Tronche (University of Pierre and Marie Curie, Paris). Genotype was confirmed using tissue obtained at ear marking. DNA extraction was achieved by incubating with 150µl 0.14M NaOH at 100^{o}C for 10 minutes, and room temperature for 5 minutes. 25µl 1M TRIS (pH 8.0) was added and the samples vortexed for 5 seconds then centrifuged for 30 seconds. 1ul of DNA was added to 0.3µl of both primers (forward: CCAGCTCAACATGCTGCACA and reverse: GCCACACCAGACACAGAGAT), and 13.4µl ReddyMix (Thermo Fisher, USA). PCR machine settings were: 1. 94^{0}C 3min; 2. 94^{0}C 30sec; 3. 65^{0}C 30sec; 4. 72^{0}C 1min; 5. Go to step 2 (5 times); 6. 94^{0}C 30sec; 7. 62^{0}C 30sec; 8. 72^{0}C 1min; 9. Go to step 6 (34 times); 10. 72^{0}C 5min; 11. 12^{0}C forever. Samples were run on a 3% agrose/TAE gel with ethylene bromide.

Viral Vectors

The DIO-ChR2-mCherry construct was kindly gifted by the Deisseroth Lab and the viral particles were produced by Vector Biolab, Philadelphia. Concentrations varied minimally with batches of virus across experiments, they ranged from 2.7-5.8*10$^{\wedge 13}$ GC/ml but were diluted down to 2x10$^{\wedge 12}$ GC/ml. The vectors were packaged in AAV serotype 2/1 vector consisting of the AAV2 ITR genomes and the AAV1 serotype capsid gene (Vector Biolab, Philadelphia).

Surgical technique and fibre optic construction

Mice, aged 10 to 12 weeks, were anaesthetised with isoflurane and placed into the stereotactic frame (David Kopf Instruments, California). 0.25% bupivacaine was injected subcutaneously beneath

the scalp. An incision into the skin was made and the head was levelled using bregma and lambda. Holes were drilled bilaterally to target the VTA. For experiments requiring viral injection only, the coordinates AP – 3.45, ML ±0.4, DV -5.05 were used. For *in vivo* optogenetic studies, to accommodate the required 10 degree ferrule angle, the coordinates were ML ±1.3, DV -4.89 (injection) -4.44 (optical fibre). 0.5 µl of purified vector was injected, bilateral, into the VTA using a 33 gauge metal needle and a 5µl Hamilton glass syringe controlled by a pump at a rate of 0.15µl/min. The needle was left for 5 minutes post injection before being slowly removed. Following this, implantable optical fibres were placed bilaterally, via the same craniotomies. The implantable optical fibres were constructed according to Sparta *et al* (Sparta et al., 2012) using 1.2mm ceramic ferrules and multimode fibre, 0.39NA, low OH, 200µm core (Thorlabs, Ely, UK). Two screws were placed dorsal to lambda and anterior to bregma to anchor a dental cement cap. Mice were kept in a heated chamber until they had recovered from anaesthesia. They were then housed with their littermates and left to recover for one to two weeks prior to the handling and habituation protocol starting. Microscopic inspection, under light anaesthesia, of a test cohort showed no damage to the ferrules by littermates after 2 weeks.

Habituation

Following surgery, a significant proportion of mice, on testing, would show no visible consumption of salt jelly following salt depletion, whilst those mice previously handled for the CPP experiment did. The importance of handling and habituation, especially following the stress of surgery is known (Deacon, 2006; Zhang et al., 2015). A handling and habituation protocol was therefore established and run alongside salt depletion. Mice were also group housed with littermates to reduce anxiety. Handling, in the test room, started 12 to 16 days before testing, breaking at weekends, and was as follows: holding the mouse in the palm for 30 seconds, with increasing time spent stroking over 3 days. Holding the mice, with the addition of partial scruffing, over 4 days. Full scruff and placement in their designated testing chamber for 15 minutes with sample test jellies, in the test day order (assigned systematically for each mouse), for five days. Sample test jellies were also placed in the home cage. All jellies were scented with almond oil wiped on the base to aid familiarisation. Jellies were omitted once the depletion protocol started. In optogenetic experiments mice were additionally tethered for the last 4 habituation days, whilst for baseline experiments mice were not scruffed. A designated test chamber was assigned to each mouse and it contained a small sample of bedding from the home cage. When furosemide injections started, the cages were rinsed daily with distilled water and the paper liner changed.

Immunohistochemistry

Mice were anaesthetised with 0.08ml euthatal 100mg/ml i.p. and perfused transcardially with approximately 30ml of 0.01M phosphate buffered saline (PBS) followed by 100ml of PBS containing 4% paraformaldehyde (4% PFA) at 4^{o}C. The cap, with attached skull and optical fibres, and brain were removed immediately and post fixed in 4% PFA. Subsequently the brains were either coronally sectioned at 70µm on a vibratome (Leica VT1000S) or cryoprotected in 30% sucrose in PBS, embedded in optimal cutting temperature (OCT) medium, frozen in isopentane at −50°C, stored at -80^{o}C and sectioned at 70µm on a cryostat (Leica CM1800, Leica Microsystems). Free floating sections were washed 4 times in PBS for 5min, then incubated with 6% normal donkey serum in 0.2% Triton X in PBS (PBS-Tx). Sections were then incubated simultaneously with primary antibodies in 2% normal donkey serum in PBS-Tx, at 4^{o}C as follows: chicken anti-tyrosine hydroxylase (1:1000; Abcam) for a minimum of 24hrs; rabbit anti-*c-fos* (1:20000; Calbiochem PC38) for a minimum of 74hrs. Afterwards sections were washed 4 times in PBS-Tx for 5min then incubated with the following secondary antibodies: AlexaFluor488 donkey anti rabbit (1:1000; Molecular Probes) and AlexaFluor633 goat anti-chicken (1:1000; Molecular Probes) or 488 goat anti-chicken (1:1000; Molecular Probes) alone for 2 hours at room temperature or 24 hours at 4^{o}C. Sections were then rinsed for 5min, first in PBS-Tx 3 times then PBS 2 times before being mounted in Vectorshield Mounting Medium (Vector Laboratories). Confocal laser scanning microscopy was performed using a Leica SP confocal microscope. Images were taken at a resolution of 1,024 × 1,024 and processed using Leica Confocal Software (Leica Microsystems), Adobe Photoshop CS3 (Adobe Systems) and ImageJ. Anatomical localization of optical fibres was assessed by examining the tracts in combination with immunolabeling for tyrosine hydroxylase to identify VTA dopamine neurons. To enable blinding, mCherry was not visualised until after excluding for fibre location.

Optogenetic setup

For optical stimulation studies implantable optic fibres were attached to 1x2 intensity division fibre-optic rotary joint (Doric Lenses Inc.) using patch cords (part code MFP_200/230/900-0.39-0.75m_FC-ZF1.25(F) Doric Lenses Inc) via a zirconia sleeve. An insulated optical fibre connected the rotary joint to a 473nm laser source (CrystaLaser and Vortran Laser Technology Inc.). Light output was adjusted by measuring the light output from the tip of an implantable optical fibre using an optical power meter. For channelrhodopsin experiments a phasic illumination pattern was used. This consisted of 8 pulses of 5ms pulse width, spaced 37ms apart, every 5s (Fig 3b) similar to previously published optogenetic stimulation studies (Adamantidis et al., 2011; Chaudhury et al., 2013; Tye et al., 2013).

Halorhodopsin experiments used a 561nm laser and a tonic illumination pattern, of 8s on 2s off (Fig 6b) (Chaudhury et al., 2013), was used with a power of approximately 2mW from the tip of ferrule.

C-fos study

7 male mice (4 DATCre$^+$ and 3 wildtype littermates) of a mixed genetic C57Bl/6 x 129Sv background underwent unilateral surgery. After a 2 week recovery period they were handled daily for 2 weeks and followed the standard habituation protocol. On the test day, mice were tethered to the laser via patchcords and placed in their designated testing chamber with a paper liner. After 15 minutes habituation, 30 minutes of phasic stimulation started. The mice were rested for 30 minutes then anaesthetised, transcardially perfused and the brains removed and sectioned at 70 µm using the cryostat. Sections were processed as above. Three sections beneath the cannula tip were analysed. From each, three areas were selected corresponding to medial, ventral and dorsolateral regions of the VTA. TH+ve cells were identified and then checked for *c-fos*. Following *c-fos* analysis mCherry expression was checked in the selected brain slices, hence the genotype was un-blinded at this point.

Open Field activity response to cocaine

This experiment was kindly performed by Dr Elaine Irvine. Each mouse was tested in a single open field arena 45cm x 45cm with 45cm walls. Naive DATCre$^+$ mice were habituated to the arena for 20 minutes and then injected i.p. with cocaine (15mg/kg) and immediately transferred back to the arena for a 60 minute test session. Their activity was recorded using a video camera suspended above the arena, interfaced with a computerised tracking (Ethovision). Distance travelled was recorded in 5 minute bins.

Electrophysiology

These experiments were kindly performed by Dr Kyoko Tossell

Slice preparation

10-12 weeks male mice (DATCre$^+$, C57BL6, treated) were deeply anaesthetised by Euthetal following isoflurane. A brain was removed by decaptation following a quick transcardialic perfusion with ice-cold artificial cerebrospinal fluid (aCSF, composition in mM, NaCl 120, KCl 3.5, NaH_2PO_4 1.25, $NaHCO_3$ 25, glucose 10, $MgCl_2$ 1, $CaCl_2$ 2) fully equilibrated with carbogen gas (95% oxygen and 5% carbon

dioxide).Two or three horizontal brain slices (190 μm thickness) encompassing the VTA were obtained using a vibratome (Leica VT1000S; Leica Microsystems, Wetzlar, Germany) and were at once incubated for 15 minutes in carbogenated NMDG-HEPES recovery solution (NMDG 93, KCl 2.5, NaH_2PO_4 1.2, $NaHCO_3$ 30, HEPES 20, Glucose 25, sodium ascorbate 5, Thiourea 2, Sodium pyrurate 3, $MgSO_4$ 10, $CaCl_2$ 0.5, pH7.3, 300mOsm, 33°C) (Zhao et al., 2011), and transferred back to aCSF. Slices were maintained in a standard custom-made maintenance chamber gently and continuously aerated with carbogen gas for at least 60 minutes at room temperature (20–22 ˚C) before use for electrophysiology.

Electrophysiology

Slices were transferred to a submersion recording chamber and were continuously perfused at a rate of 2-4 ml/min with fully oxygenated aCSF at 32°C. Neurons were visualized using infra-red differential interference contract (IR-DIC) under an upright microscope (Olympus BXWI 51, Japan) equipped with a 40x objective (0.8 numerical aperture), an IR filter, DIC optics and a charge coupled device (CCD) video camera (Watac). For visualising recorded neurons, 0.1% neurobiotin (Vectorlab) was added to all intercellular solutions.

Whole-cell patch-clamp recordings were performed with a Multiclamp 700B amplifier (Molecular Devices, CA) and an Axopatch 200A amplifier (Axon Instruments). The signals were sampled at 20 kHz and low-pass filtered at 1 kHz. Series resistance (Rs) and input resistance (Rin) were frequently monitored throughout the experiments via a 10mV, 250ms hyperpolarizing step. Any large changes in holding current or noise characteristics were taken as early signs of cell loss and recordings were terminated. Experiments were also terminated if Rs exceeded 35 MΩ or if Rin changed more than 15% after break in the whole-cell mode. Rs (typical values of 10-30 MΩ) was compensated by 60-70% in the majority of the experiments. Membrane capacitance (Vuong et al.) was measured under voltage clamp at -50 mV using a hyperpolarizing 10 mV, 250 ms step. C_m was measured from the change in membrane charge taken from the integrated capacity transients (pClamp, Molecular Devices). All potentials cited here have not been corrected for liquid junction potentials (estimated using pClamp calculator as 9.2 mV). Slices were incubated in drug cocktails for minimum 15 minutes prior to recording.

Optogenetics –acute brain slices from DATCre^{+} mice expressing mCherryChR2

VTA DA neurons expressing ChR2 were identified by visualizing mCherry. The whole-cell recording electrode (5-7 MΩ) was filled with an internal solution containing (in mM): K-Gluconate 140, KCl 5, HEPES 10, EGTA 0.1, $MgCl_2$ 2, MgATP 2, NaGTP 0.2 (pH 7.3-7.4, 280-285 mOsm). A blue light (470nm) was delivered by TTL-control from a microscope-mounted LED to the entire field through the objective. After the achievement of stable current clamp, rapid flash of light 5ms (25Hz) with inter-stimulus interval 5s was given for 4 times at the 60s inter-sweep interval. The light intensity was adjusted according to the magnitude of its response.

Optogenetics –acute brain slices from DATCre^{+} mice expressing eNpHR3.0-EYFP

VTA DA neurons expressing eNpHR3.0 were identified by visualizing YFP. The whole-cell recording electrode (5-7 MΩ) was filled with an internal solution containing (in mM): K-Gluconate 140, KCl 5, HEPES 10, EGTA 0.1, $MgCl_2$ 2, MgATP 2, NaGTP 0.2 (pH 7.3-7.4, 280-285 mOsm).The yellow light (585 nm) was delivered by TTL-control from a microscope-mounted LED to the entire field through the objective. After the stable current clamp was achieved, +75pA current step (12s) was given and 2 sets of 8s continuous light stimulation was applied at 2s interval. The light intensity was adjusted according to the magnitude of its response.

Conditioned place preference

Mice were housed in pairs or trios following surgery. After the recovery period they were handled and scruffed for a week. A biased conditioned place preference (CPP) was performed based on Tsai et al., (2009) but with 4 days of conditioning rather than 2 days. A three compartment CPP setup was used (Med Assoc. Inc.), the white and black box were scented with lemon and ethanol respectively. Day 1, mice were placed in the central grey area for 2 min, gates were then opened allowing free exploration of all 3 boxes for 15 minutes. Time spent in each area was recorded and their chamber of preference noted. On the first day of conditioning (day 2), the mice were placed in one box and the following day the other box for 30 minutes. They were tethered in both boxes but only stimulated in the box they showed least preference for on day 1. Light output was adjusted to 2mW from the tip. This conditioning was continued for days 4 and 5. On day 6 the mice were tested for preference over 15 minutes without stimulation. Preference for chambers during the first and the last stimulation-free test day were analysed to produce the conditioned place preference result. Average locomotor activity, using beam break data, during the stimulation days with respect to the

non-stimulation days for each mouse (days 2-5) was analysed to assess whether stimulation increased locomotor activity.

ChR mediated dopamine stimulation and sodium appetite

Following the CPP experiment the same cohort of mice were transferred to low salt diet and salt depleted according to protocol. Henceforth in my thesis, salt depletion and canteen testing is always carried out on a cohort of mice that have undergone no previous experiments. Furosemide dose in the first test was 10mg/kg. Subsequently, one cohort was abandoned since both DATCre^{+} and control groups failed to consume during canteen testing. Therefore habituation was increased, jellies dishes were scented with almond oil underneath and the furosemide dose was increased to 20mg/kg. This dose has then been used throughout my thesis.

On the test day mice were tethered and placed in their designated chamber. After 10 min habituation laser stimulation began and the canteen test started five minutes later.

Exclusion criteria

Occasionally, despite adequate weight loss, indicating salt depletion, and full habituation and handling, some mice did not consume in the first 10 minutes. When the mice were fully habituated, this applied to 1-2 mice per group (5-10%). There was no significant difference between the DATCre^{+} and control groups. As there was no consumption, and the measured outcome was the relative intake of the three concentrations, no meaningful data could be obtained from these mice. They were therefore excluded. Once the lids from the jellies were removed evaporation occurred. A control study in which a set of jellies were placed alongside the test cages showed that this loss in weight was ≤ 0.005g per 10 minutes. A weight loss of ≤0.005g in all three jelly pots was taken as no consumption and the mouse was excluded.

Location of optic fibres was assessed post mortem and the mouse excluded if the tip was not located above the VTA. After exclusion on this criteria the mice's genotype was unblinded and mCherry/Yfp expression was examined. DATCre^{+} mice were excluded if there was no expression.

3.3. Results

Stereotactic injection of the viral vector carrying the channelrhodopsin/mCherry construct resulted in widespread expression as shown by colocalisation of mCherry and TH (Fig. 3.2a). This expression permitted *in vivo* activation of dopamine neurons upon thirty minutes of phasic blue light illumination (Fig 3.2b). Neuronal activation was characterised by the expression of the depolarisation marker, *c-fos*, in dopamine neurons (Fig. 3.2c). Following 30 minutes of phasic blue light illumination, *c-fos*/TH co-expression was greater in DATCre^{+} mice compared to wild type controls (Fig 3.2d). *Ex vivo* studies confirmed the precise temporal nature of this set up (Fig. 3.2e). Every 5ms blue light illumination resulted in the triggering of an action potential.

Testing DATCre^{+} mice against wild type littermates showed that there was no behavioural phenotype associated with the expression of Cre in dopamine neurons with regard to salt appetite (3.3a and b). When the dopamine system was stimulated with cocaine there was also no difference in the locomotor response between the DATCre^{+} and DATCre^{-} mice (Fig. 3.3c).

Alongside testing the physiological response of ChR2 expression in dopamine neurons *ex vivo* I also wished to ensure that the setup was capable of inducing a behavioural effect *in vivo*. I therefore replicated a CPP experiment previously published (Tsai et al., 2009). My results showed that after training, the DATCre^{-} control group did not significantly change their preference for the chamber in which blue light stimulation occurred (Fig. 3.4a). However, the DATCre^{+} increased the time they spent in the stimulated chamber after four days of conditioning (Fig. 3.4b). The CPP apparatus includes beam break equipment which enabled analysis of the effect that optogenetic stimulation of dopamine neurons had on movement. This showed that the stimulation protocol used was not associated with increased activity (Fig. 3.4c).

Once it was confirmed that the expression of ChR2 in VTA dopamine neurons and the stimulation protocol was sufficient to elicit a CPP, the same cohort of mice was then depleted of salt and the effect of phasic dopamine stimulation on salt appetite was measured.

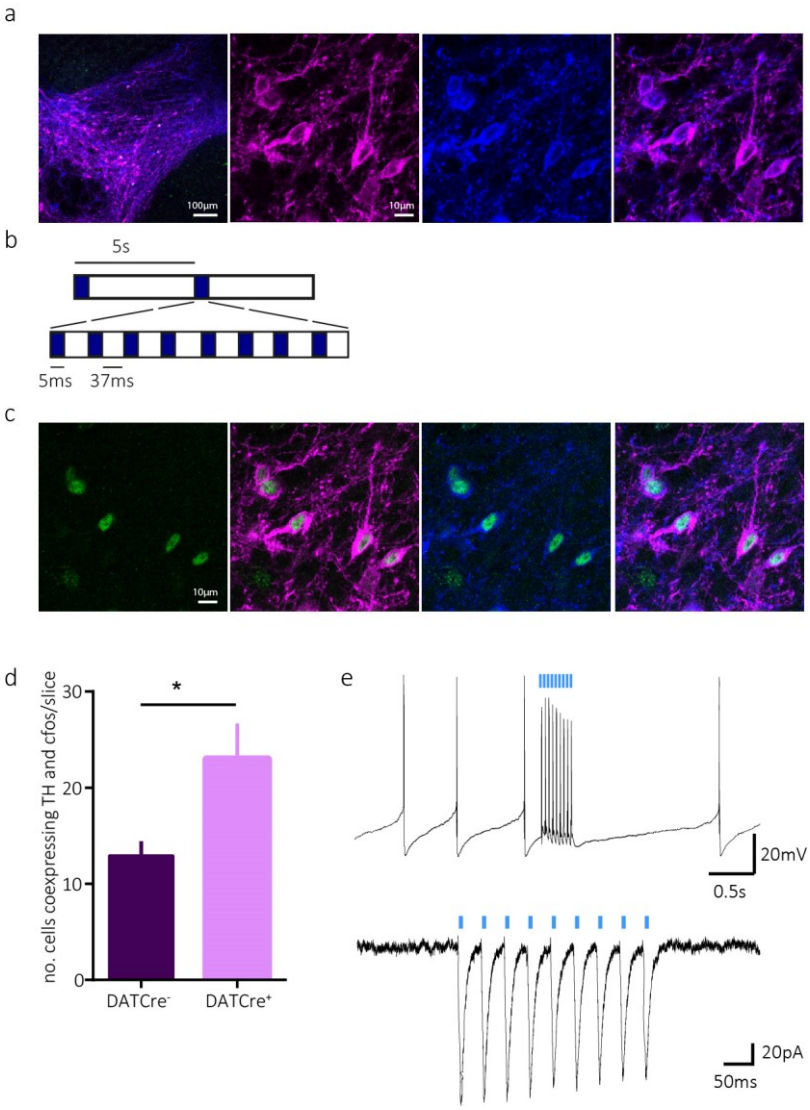

Figure 3.2: ChR2 expression in dopamine neurons

a. Confocal images show widespread ChR2-mCherry (magenta) expression in TH positive cells (blue) in the VTA. **b.** Phasic blue light stimulation consists of eight 5ms flashes every 5s. **c.** 30 minutes of phasic, *in vivo* illumination of the VTA results in dopamine neuron activation as shown by staining for the depolarisation marker *c-fos* (green). **d.** Quantification of TH cells expressing *c-fos* showed a significant increase in DATCre^{+} mice (n=4) compared to controls (n=3) ($p<0.05$). Error bars represent SEM throughout. **e.** Recordings of dopamine neurons in acute brain slices, every 5ms flash results in an action potential driven by an inward current following ChR2 activation.

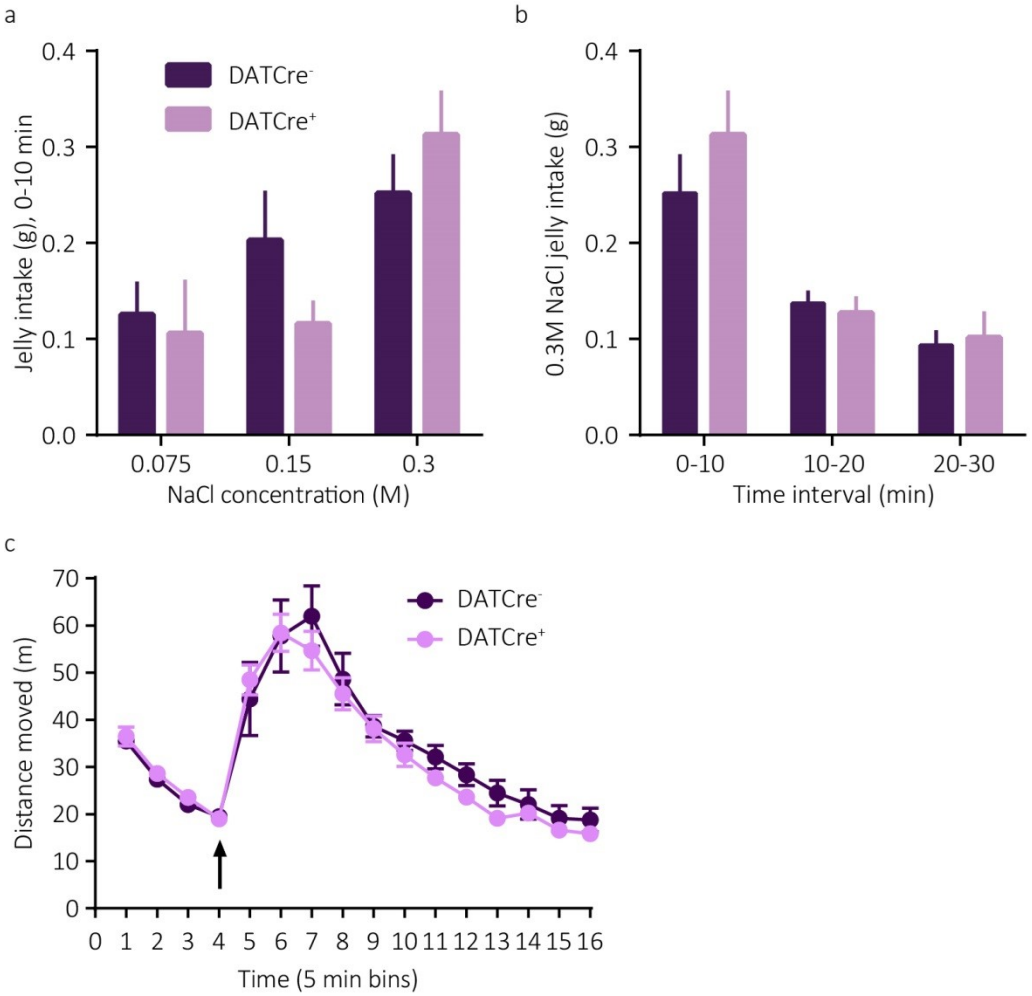

Figure 3.3: Exclusion of Cre related phenotype

Heterozygous DATCre^{+} (n=7) mice show no difference to their wild type littermates (n=11) with regard to salt appetite; **a.** Intake of all salt jelly concentrations in the first 10 minutes (concentration x genotype $F(2, 32)=1.064$ n.s. concentration $F(2, 32)=5.791$ $p<0.01$, genotype $F(1, 16)=0.3285$ n.s.). **b.** Intake of the 0.3M NaCl jelly over 30 minutes (time x genotype $F(2, 32)=0.8807$ n.s. time $F(2, 32)=24.81$ $p<0.0001$ genotype $F(1, 16)=0.5296$ n.s.). **c.** DATCre^{-} (n=7) and DATCre^{+} (n=8) mice increased their activity in open field testing following cocaine (time x genotype $F(15, 195)=0.64744$ n.s. time $F(15, 195)=56.23$ $p<0.0001$ genotype $F(13, 195)$ n.s.).

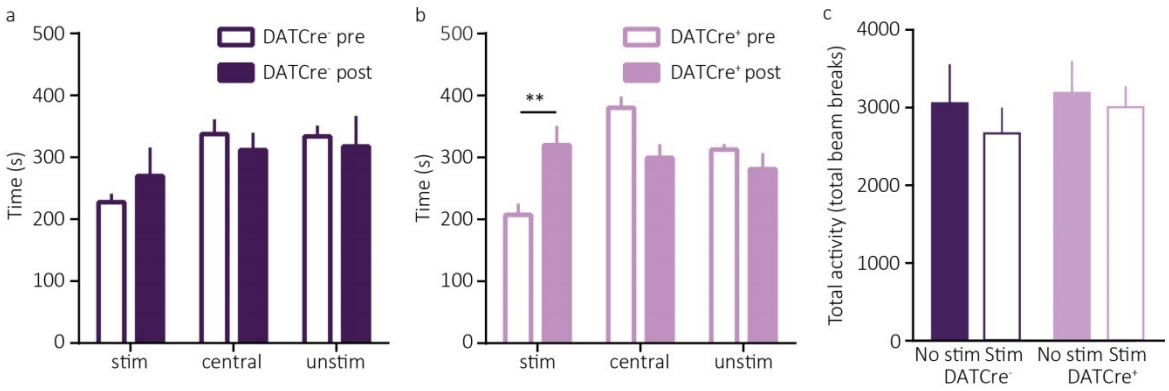

Figure 3.4: Phasic optogenetic stimulation of VTA dopamine neurons elicits a CPP

a. DATCre$^-$ mice (n=6) showed no preference for the chamber where they had previously received stimulation (Stimulation; Session x Stimulation F'S<2.9 p's>0.15 n.s.). **b.** DATcre$^+$ mice (n=9) showed a significant preference for the chamber in which they had previously received stimulation (session x stimulation F (1,8)=7.0 p<.05, pairwise comparisons revealed this was specific to the stimulated chamber, pre vs post session p<.01). **c.** Optogenetic stimulation did not change locomotor activity as measured by beam breaks during the stimulation days of the biased CPP test (stimulation; stimulation x genotype all F's<1 p's>0.4 n.s.).

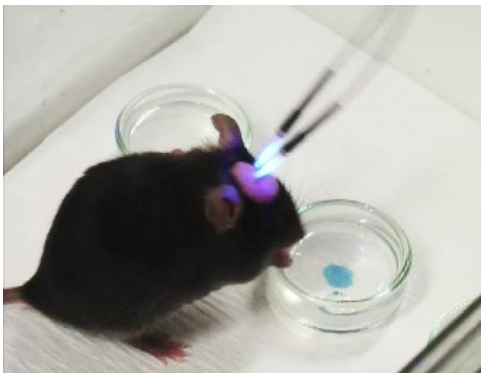

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Figure 3.5: Phasic stimulation of dopamine neurons during salt jelly canteen testing

DATCre^{+} mouse eating from the central, 0.075M NaCl jelly tub during *in vivo* phasic stimulation of dopamine neurons.

Unexpectedly, this resulted in a reduction of intake of 0.3M salt jelly (Fig. 3.6). The effect was only seen in the first 10 minutes which is when the highest consumption usually occurs, but did not lead to compensatory higher intakes later in the experiment. In the first 10 minutes the reduction was specific for the highest concentration and there was no overall reduction in intake by the DATCre^{+} group. This concentration specific effect is shown by the interaction between concentration and genotype. Subsequent multiple comparisons showed a significant reduction in 0.3M concentrations (Fig. 3.6c). To ensure the reliability of my results I repeated the salt appetite experiment in another cohort of mice.

The results were replicated (Fig. 3.6 d to f) with a specific reduction in intake of the highest concentration salt jelly. I next wanted to check that my results were due to modulation of VTA dopamine neuronal activity rather than neuron damage. Hence I repeated the test the following week. The mice were tethered but not stimulated. The unstimulated test showed no difference between the two groups so there had been no obvious damage done to the neuronal network

involved either by viral expression or previous optogenetics stimulation.

It has been reported that prolonged ChR-mediated depolarisation may result in a depolarisation block and hence inhibition (Herman et al., 2014), the results of the CPP and *c-fos* experiment indicate that the stimulation protocol used resulted in activation. However, I was interested to see if direct inhibition of dopamine neurons resulted in the same result or the opposite. I therefore went on to test the effect of inhibition using halorhodopsin.

Immunohistochemistry showed expression of eNpHR3.0-EYFP in dopamine neurons following intra VTA injections of the viral vector (Fig 3.7a). The dynamics of the halorhodopsin pump requires a recovery period therefore, continuous illumination was not used and instead illumination was for 8 second intervals separated by 2 seconds (Fig. 3.7b) in line with protocols in published data (Chaudhury et al., 2013; Tye et al., 2013). *ex vivo* electrophysiology confirmed that this expression correlated with the presence of functioning halorhodopsin. Exposure to intermittent yellow light showed a reduction in the firing rate of eYFP expressing dopamine neurons during illumination (Fig. 3.7c).

Testing salt appetite during halorhodopsin mediated inhibition of dopamine neurons produced results similar to ChR2 mediated stimulation (Fig. 3.8). Although analysis of the first 10 minutes does not show an interaction between genotype and concentration, as with the ChR results, this is likely

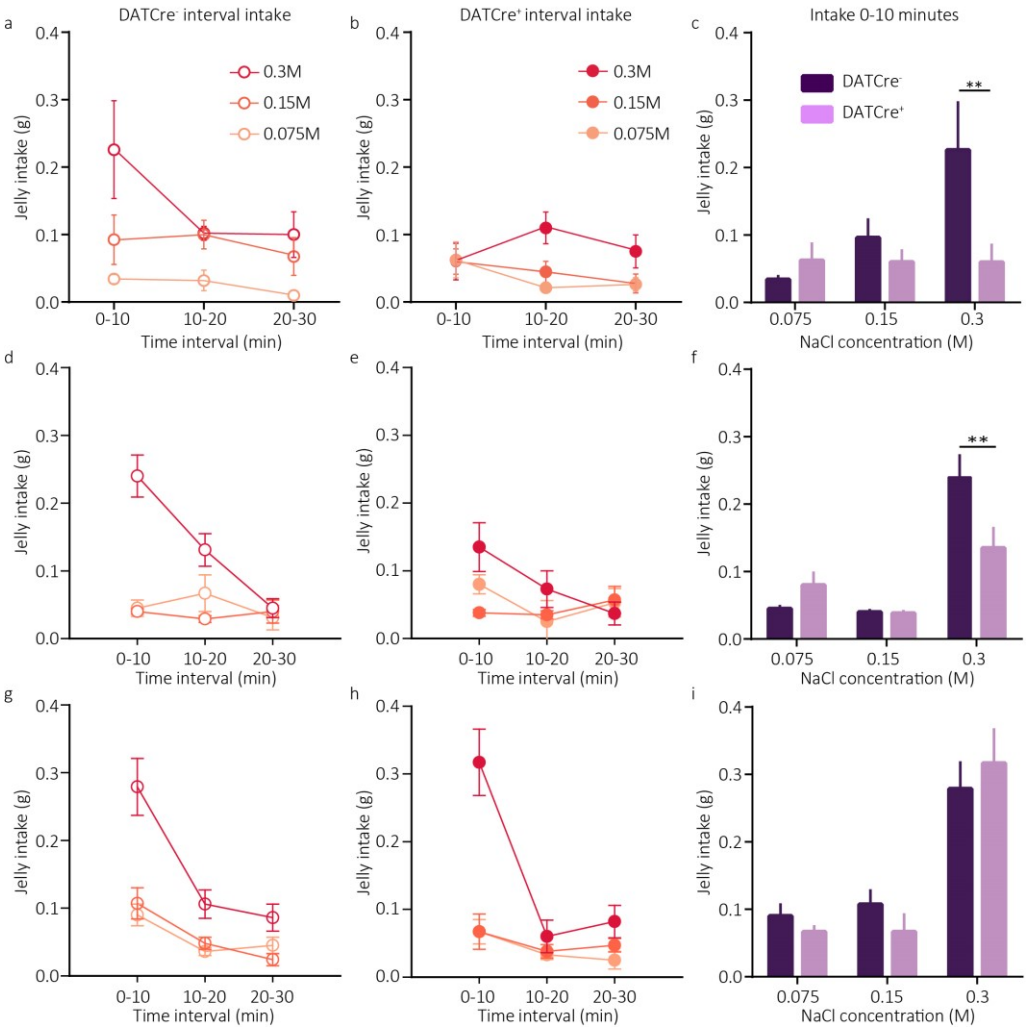

Figure 3.6: Phasic stimulation of VTA dopamine neurons during salt jelly canteen testing

a. and **b.** DATCre$^+$ mice reduced their intake of 0.3M NaCl jelly in the first 10 minutes. **c.** The effect was specific for 0.3M NaCl with no difference in intake of the other jellies in the first 10 minutes. DATcre$^-$ (n=5), DATcre$^+$ (n=8) (genotype x concentration $F(2,22)=3.87$ $p<0.05$, pairwise comparisons 0.3M NaCl DATcre$^-$ vs DATcre$^+$ $p<0.01$). Results were replicated in the second cohort **d.** DATcre$^-$ (n=8) and **e.** DATcre$^+$ mice (n=6) differed in their intake of the different concentration salt jellies (time x genotype x concentration $F(4, 48)=3.6$ $p<.05$. Main effects of Time and Concentration and Time x Concentration). **f.** A selective reduction in intake of the high concentration salt jelly occurred during the first 10 minutes of the test session in the DATcre$^+$ mice (concentration x genotype $F(2,24)=7.1$ $p<0.005$, pairwise comparisons 0.3M salt DATcre$^-$ vs DATcre$^+$ $p<.05$). When tested a week later in the absence of stimulation, **g** and **h**, mice preferentially consumed the highest concentration salt jelly (concentration $F(1.3, 16.1)=40.6$ $p<.001$) and no differences in intake were observed between genotype groups across the session (time x genotype x concentration $F<1.8$ $p>0.15$ n.s., concentration x genotype $F<1$ $p>0.9$ n.s.) nor during the first 10 minutes of the session **i.**

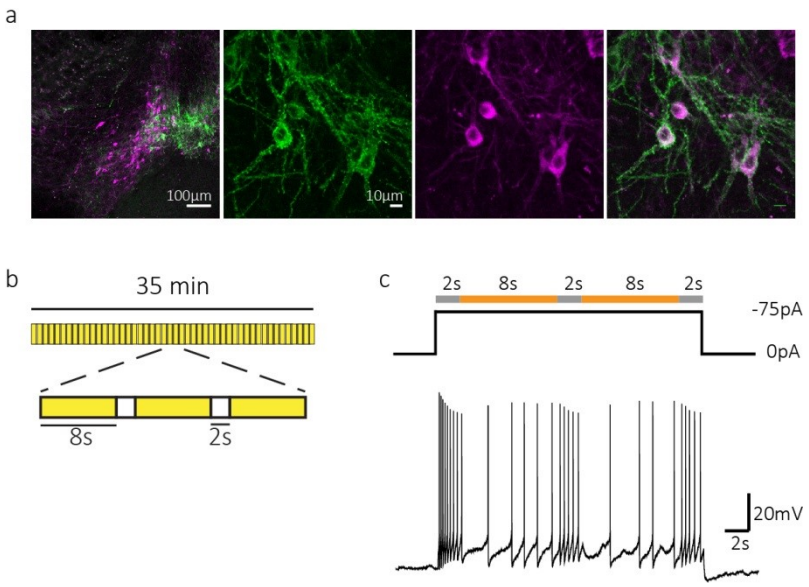

Figure 3.7: eNpHR3.0-EYFP expression in dopamine neurons

a. Confocal images show widespread eNpHR3.0-EYFP expression (green) in TH positive cells (magenta) in the VTA. **b. I**ntermittent yellow light illumination consisted of alternate 8 seconds on 2 seconds off. **c.** Recordings of dopamine neurons in acute brain slices, every 8s illumination results in a reduction in action potentials.

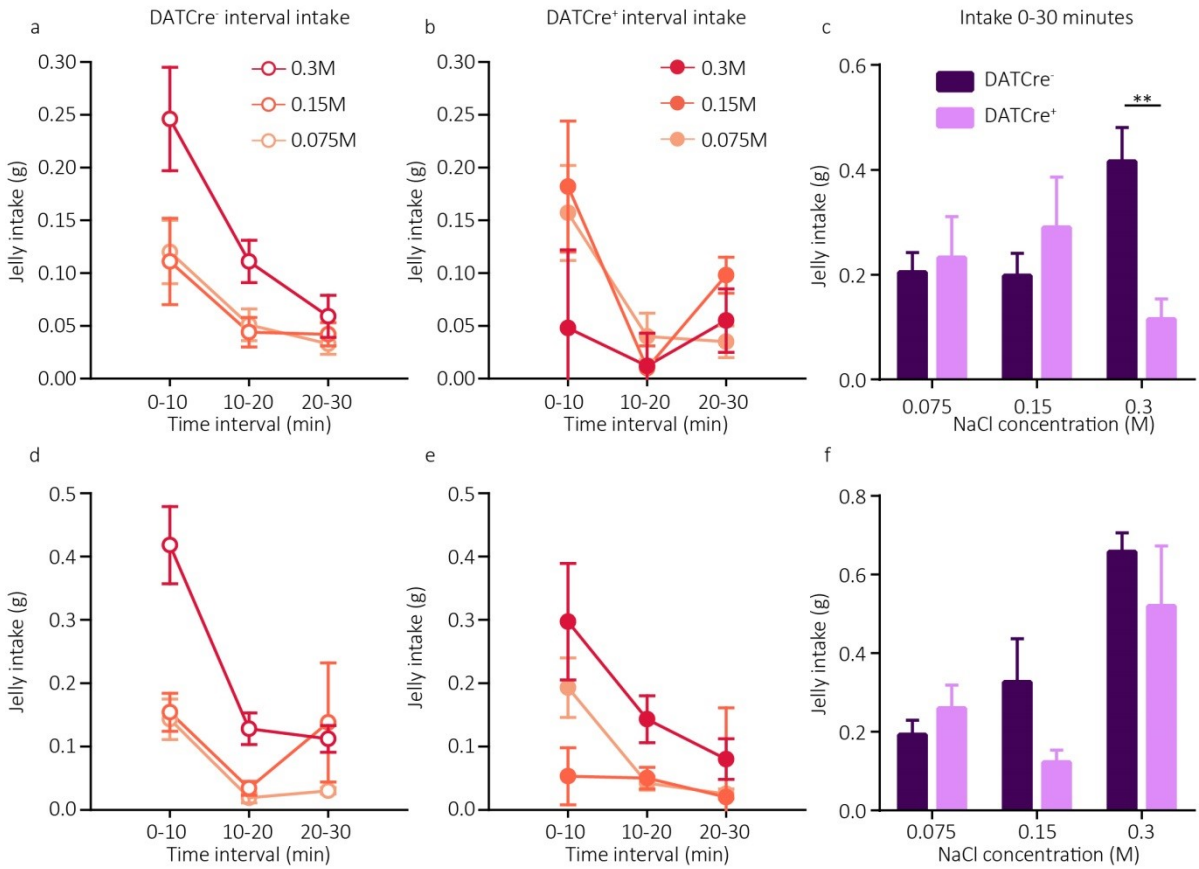

Figure 3.8: Optogenetic inhibition of VTA dopamine neurons during salt jelly canteen test

Optogenetic inhibition of VTA dopamine neurons significantly decreased intake of the highest concentration salt jelly in **b.** DATcre^{+} (n=4) mice with respect to **a.** DATcre^{-} mice (n=9), (concentration x genotype $F(1,22)=4.2$ $p<.05$). The selective reduction in intake of the high concentration salt jelly persisted throughout the 30 min session, (time x concentration x genotype $F(4,44)=1.9$ $p>0.1$ n.s.). **c.** The reduced intake of salt jellies in the DATcre^{+} mice was selective to the high concentration jelly only, (pairwise comparisons; 0.3M salt jelly DATcre^{-} vs DATcre^{+} $p<0.05$). **d.** and **e.** When tested a week later under a salt appetite in the absence of stimulation, no differences in intake were observed between groups, across the session (time x genotype x concentration $F<1$ $p>0.7$ n.s., concentration x genotype $F<1.2$ $p>0.3$ n.s.). **f.** All mice preferred the high concentration salt jelly, (concentration $F(2,22)=10.0$ $p<.005$). Data represented as means ± SEM.

to be due to insufficient animal numbers following exclusions. Longitudinal analysis over the full 30 minutes shows an interaction, and post hoc testing shows a significant difference in 0.3M NaCl intake in the first 10 minutes between DATCre^{+} and DATCre^{-} controls. The selective reduction in 0.3M NaCl jelly intake is also revealed by analysis of the total intake over 30 minutes (Fig. 3.8c).

3.4. Discussion

During the development of the BAC-DATiCre line, Turiault et al (Turiault et al., 2007) designed the BAC transgene so that there would be no undesired expression of DAT proteins. To rule out the existence of any problems they tested the DATCre^{+} males with regard to weight, muscular strength, locomotion, anxiety-like and despair-like behaviour but not feeding nor response to dopamine system stimulation. In this chapter I have shown that heterozygous DATCre^{+} male mice have a normal activity response to i.p. cocaine and shows normal consummatory behaviour in the context of salt appetite. The use of this transgenic mouse line to investigate salt appetite is therefore appropriate.

Brown et al (2010) have used the BAC-DATiCre line for optogenetic experiments whilst other papers have used tyrosine hydroxylase (TH): internal ribosomal entry sire (IRES)-Cre transgenic mice (Tsai et al., 2009) or a knock-in DAT-Cre line (Domingos et al., 2011). There has been shown to be different cellular expression of Cre in mice Cre lines targeting dopamine neurons (Vuong et al., 2015). Dopamine neurons vary in their individual expression of DAT and TH with some topographical difference (Björklund and Dunnett, 2007), and transgenic lines may express abnormal behavioural phenotypes. Replication of optogenetic experiments between the transgenic lines can therefore not be assumed. The CPP and *c-fos* experiments confirm that the optogenetic set up was successful with regard to the fact that dopamine neurons were stimulated and that stimulation was not aversive; aversive stimuli would drive a conditioned place aversion (Tan et al., 2012). This is important since there are topographical differences between dopamine neurons and their response to aversive stimuli (Brischoux et al., 2009; Lammel et al., 2011). I was targeting the mid to posterior, lateral, dorsal VTA; in rats dorsal VTA dopamine neurons are inhibited by noxious footshocks whist dopamine neurons in the ventral VTA are phasically excited (Brischoux et al., 2009).

Strikingly, ChR stimulation led to a decrease in salt intake. That the effect was concentration specific indicates that this was not due to the generation of a state unconducive to consumption by, for example, the generation of fear, anxiety or movement-related problems. This is further supported by

the observation that 0.3M salt consumption was no different from control after 10 minutes when CHR stimulation was ongoing. The results suggest that the consequence of VTA dopamine neuron stimulation interfered with the process by which salt appetite leads to higher value being assigned to the high salt jelly which in turn provides the motivation to consume. Literature regarding the role of dopamine in reward suggests that tonic dopamine reports an overall reward rate whereas phasic dopamine signals a reward. The phasic signal then updates the overall reward rate (Niv et al., 2007). The overall reward rate then modulates the vigour of movement (Beierholm et al., 2013). My results cannot be explained in the context of this model. This may be due to the fact that this model of dopamine function was constructed in the context of free-operant tasks which is very different from my experimental design. My experimental design is more akin to free reward consumption. This design has been used to study the role of dopamine neurons in alcohol consumption. Studies have shown that in alcohol withdrawal there is a marked reduction in spontaneous firing rates of dopaminergic cells in the VTA (Bailey et al., 2001) and optogenetic stimulation of VTA dopamine neurons during voluntary ethanol drinking sessions attenuated intake (Bass et al., 2013). This suggests that in alcohol withdrawal there is inhibition of dopamine neurons and consequently a reduction in striatal dopamine. Optogenetic stimulation of dopamine neurons eliminates the withdrawal state, possibly via increased striatal dopamine. Salt appetite is triggered by AII and an ACE inhibitor reduces AII synthesis. Chronic, peripheral administration of an ACE inhibitor (perindopril), which crosses the BBB, results in an increase in striatal dopamine (Jenkins et al., 1997), hence conversely, AII would be expected to lead to a reduction in striatal dopamine. Therefore, production of AII stimulated by salt depletion may lead to a reduction in striatal dopamine and hence a withdrawal state similar to alcohol. This withdrawal state would drive ingestion of hypertonic saline leading to dopamine release in the striatum (Cone et al., 2016) and hence amelioration of the withdrawal state. This explanation does not designate dopamine as a satiety signal. Satiety would lead to the removal of inhibition which results in striatal dopamine reduction, and also disruption of the pathway that results in hypertonic saline ingestion stimulating dopamine release. In sham feeding satiety is not achieved despite activation of the mesolimbic system (Voorhies and Bernstein, 2006). Optogenetic stimulation of dopamine neurons immediately prior to, and during, expression of salt appetite, results in striatal dopamine release and therefore a resultant reduction in the withdrawal state. Hence, there is attenuated intake of high concentration salt jelly. Some ingestion still occurs so satiety will be achieved. The attenuation of ethanol was seen only during tonic stimulation of dopamine neurons not phasic, so the study by Bass *et* al does not completely complement my results. However, it should be noted that the phasic stimulation protocol used a frequency of 50 Hz. This is much greater than the natural frequency of dopamine neuron burst firing

and has been shown not to be ideal in optogenetic studies of dopamine neurons (Adamantidis et al., 2011). Also, *in vivo* phasic firing of dopamine neurons in response to reward is specific with regard to timing of the RPE. When replicating dopamine activity using optogenetics this timing is important (Hamid et al., 2016). This is not true for tonic firing. Optogenetic activation is synchronised therefore studies are only able to replicate the constant, low frequency characteristics of tonic firing compared to the intermittent, high frequency of phasic firing. The synchronised nature of optogenetically controlled tonic firing may result in responses similar to timed, reward related phasic firing.

I found that halorhodopsin-mediated inhibition of dopamine neurons also reduced consumption. McCutcheon *et al* (McCutcheon et al., 2014) showed that somatic, halorhodopsin-mediated inhibition of dopamine neurons is capable of supressing dopamine transients in the NAc which had been induced by cocaine /raclopride cocktail. The suppression was not complete and dopamine transients were detected in some rats but at a lower frequency. When they were detected the amplitude was not affected by the laser. My results therefore cannot be explained by somatic, halorhodopsin-mediated inhibition of dopamine neurons being incapable of suppressing dopamine transients in the NAc. This is important since dopamine transients can triggered independent of dopamine neuron firing (Cachope et al., 2012). One explanation for my results may be that during the 2 seconds of light off there was compensatory dopamine activity, subsequent dopamine release in the striatum may therefore replicate the effect of ChR stimulation. Brischoux *et al* noticed significant *in vivo* excitation in dopamine neurons following inhibition generated by a noxious stimulus. This indicates that rebound excitation may be the normal response of dopamine neurons to intermittent inhibition. Another factor is the possibility that hyperpolarisation mediated by halorhodopsin may engage hyperpolarisation-activated channels resulting sometimes in rebound action potentials (Allen et al., 2015). A “rebound” effect has been seen in hippocampal neurons, thought to be due to changes in the $GABA_A$ receptor reversal potential (Raimondo et al., 2012). This may be physiological and not necessarily specific to halorhodopsin induce hyperpolarisation. The stimulation protocol was used by Chaudhury et al (Chaudhury et al., 2013) and the ex vivo electrophysiology studies in their supplementary material fails to show rebound excitation. This study, and our ex vivo study, were performed during evoked firing, the results can therefore not necessarily be extrapolated to the *in vivo* situation. The stimulation protocol for halorhodopsin necessitates prolonged illumination. There is therefore the potential for off-target effects due to heating. The use of a $DATcre^-$ control group should eliminate this confound unless expression of the halorhodopsin protein rendered dopamine neurons more sensitive to thermal injury . Alternatively this result may be genuinely due to inhibition of dopamine neurons in a similar way to which GABA

stimulation, and hence dopamine inhibition, led to a reduction in free reward consumption (van Zessen et al., 2012).

In summary my optogenetic experiments showed that both optogenetic mediated excitation and inhibition of VTA dopamine neuron during salt seeking behaviour led to a reduction in intake of the favoured high salt jelly. I therefore decided to repeat these experiments using an alternative method to excite and inhibit dopamine neurons. Hence, I set up and used chemogenetics to investigate the above results.

Chapter 4 Chemogenetic modulation of dopamine neurons and the effect on salt seeking behaviour

4.1. Introduction

In the previous chapter I showed that phasic, optogenetic excitation of dopamine neurons attenuated consumption of the high concentration salt jelly during salt seeking behaviour. In this chapter I sought to compliment my optogenetic experiments by using an alternative technique, chemogenetics, to excite and inhibit dopamine neurons.

Whilst optogenetics uses light and genetics to regulate cellular activity, chemogenetics uses genetics and small drug like chemical actuators (English and Roth, 2015). The armoury of chemogenetic technology is expanding rapidly. However, the most established genetic construct encodes for Designer Receptors Exclusively Activated by Designer Drugs (DREADDs). These are engineered G-protein-coupled receptors (GPCRs) that can be activated by inert small molecules. GPCRs are integral membrane proteins that mediate nearly all physiologic processes in the body by responding to a variety of ligands resulting in the modulation of various downstream signalling pathways (Abbott et al., 2013). The DREADDs that I use are derived from the human muscarinic acetylcholine receptors (hMDREADDs). Created by directed molecular evolution, the resultant mutated receptors do not respond to the endogenous ligand, acetylcholine, but are activated by the clozapine metabolite clozapine-N-oxide (CNO) (Armbruster et al., 2007). The M3-muscarinic DREADD (hM3Dq) has 2 mutations Y149C and A239G, and is selective for the Gαq-mediated signalling pathway. CNO binding results in a rise in intracellular calcium, this triggers burst firing of neurons (Sternson and Roth, 2014b). The M4-muscarinic DREADD (hM4Di) has mutations Y113C and A203G, and is selective for the Gαi-mediated signalling pathway. Binding of CNO leads to activation of the G-protein inwardly rectifying potassium (GIRK) channels, resulting in neuronal silencing (Armbruster et al., 2007). It also leads to a reduction in cAMP which will result in further cellular events. The potential consequences of these off target effects are not fully characterised but may be very important in studies of long duration and repeat testing. For example, cAMP plays a pivotal role in the intra-cellular pathways that lead to synaptic plasticity (Anderson and Pierce, 2005). Chemogenetic modulation may therefore interact with this process. As with optogenetics, the strength of DREADD technology is the ability to target specific cell types using a variety of different technologies. Although it must be noted that the cellular response to GPCR signalling pathway activation (both M3 and M4) is cell type specific. This has allowed the technology to be used in a variety of biological systems outside of

neuroscience, for example, pancreatic beta cells (Guettier et al., 2009), endothelial cells (Mikelis et al., 2015) and lymphoma cells (O'Hayre et al., 2015), but confers an importance to verifying the change in activity profile following CNO application since different neuronal types may not respond in the same manner (Sternson and Roth, 2014b).

An additional strength of DREADD is the ease of CNO administration. Numerous papers have shown a central effect of peripherally administered CNO in DREADD expressing experimental animals (reviewed in Wess et al 2013), indicating that CNO must penetrate the blood brain barrier (Wess et al., 2013). After a single intraperitoneal (i.p.) injection of CNO (1mg/kg) in mice, plasma levels peak after 15 minutes and are very low after 2 hours (Guettier et al., 2009). The biological effect though, may persist beyond the plasma availability, lasting for 6 to 10 hours (Alexander et al., 2009). CNO may also be given in the drinking water. Systemic administration allows for a longer lasting effect on neuron activity but trades off the precise temporal control of optogenetics (Sternson and Roth, 2014a). Systemic administration also enables targeting of larger structures whilst optogenetics is limited to the small area of illumination.

Studies using chemogenetics to directly modulate VTA dopamine neurons are less numerous than optogenetics. Activation of dopamine neurons expressing M3 results in hyperactivity (Wang et al., 2013) and may help to mitigate against the motor symptoms in mouse models of Parkinson's Disease (PD). To achieve this chemogenetic technology, gene therapy and tissue engineering has been combined to gain remote control of induced dopaminergic neurons transplanted into PD animals (Dell'Anno et al., 2014) (Aldrin-Kirk et al., 2016). These studies illustrate the possible therapeutic potential of chemogenetics.

Those studies focusing on reward and feeding have often combined chemogenetics with Cre expressing viral vectors, or local application of CNO to target neurons projecting to the VTA or specific VTA projections. This enables the interrogation of distinct pathways, which incorporate VTA dopamine neurons, and their role in certain behaviours. For example, since there is no contra-lateral VTA projection of VP neurons, simultaneous injection of a DIO-Syn-hM4Di-mCherry AAV construct (which is Cre dependent) into the left VTA of a TH Cre^{+} rat and contralateral injection of a Syn-hM4Di-HA-GFP lentivirus (neuronal specific not Cre dependent) into the right rostral ventral pallidum (RVP) enables isolation of VTA dopamine neurons from RVP inputs upon systemic CNO administration. CNO does not affect the left RVP and its projections, including input onto non-dopaminergic neurons in the left VTA. The right VTA has uninhibited dopamine neurons but does not receive input from the VP. In a study establishing the role of RVP to VTA projections in cocaine

seeking behaviour, projections onto dopamine and non-dopamine VTA neurons may thus be functionally separated (Mahler et al., 2014). Targeting subtypes of VTA dopamine neurons, with respect to their axonal projection, may be achieved by injecting a virus, which infects axonal terminals and is then trafficked retrograde to the cell body, for example, canine adeno-virus-2 (CAV-2), into the distal projection site such as the NAc (Boender et al., 2014). AAV-hSyn-DIO-hM3D(Gq)-mCherry is then injected into the VTA. Since the hM3D construct is Cre dependent, expression is limited to those which send axonal projections to the NAc and have therefore been infected by the CAV-2 and hence express Cre. Systemic CNO will then cause activation of those VTA neurons projecting to the NAc. This modulation is not cell specific, but by combining with pharmacology the neurotransmitter involved may be identified. By using these techniques Boender *et al* showed that activation of VTA neurons projecting to the NAc increased progressive ratio performance, a measure of motivation, as well as locomotor activity. The latter only when high doses of CNO were administered. In addition to using chemogenetics to modulate neuronal activity, and thereby elucidate networks and interrogate the behavioural function, chemogenetics has also been utilised to identify intracellular signalling pathways in VTA dopamine involved in anhedonia (Zhong et al., 2014). CNO administration led to a rise in cAMP levels in VTA dopamine neurons and thereby reversed the behavioural deficit induced by Cdk5 deletion. The results suggest that Cdk5 in the VTA regulates cAMP/PKA signaling and it is by this pathway that loss of Cdk5 function results in depression-related behaviours.

The mesolimbic system plays an important role in appetite. The longer lasting effect of chemogenetics lends itself to feeding studies. It has therefore been used in studies intent on delineating neural pathways and neurotransmitters involved in overeating. For example, using chemogenetics to modulate glucagon-like peptide-1 (GLP-1) secreting neurons which project from the NTS. Activation of these neurons in unfasted mice led to a decrease in high fat diet (HFD) intake but had no effect on normal chow. Inhibition led to a reduction in intake of HFD (Wang et al., 2015). Administration of a GLP-1R antagonist, during chemogenetic modulation, blocked the effect and hence indicates that GLP-1 is the neurotransmitter responsible for the effect. NTS GLP-1 secreting neurons project to a number of sites including the VTA. The role of the mesolimbic system, in the GLP-1 control on appetite, was characterised by local application of CNO to the VTA. This replicated the results when M3 was expressed but not M4. Results from *ex vivo* electrophysiology studies imply GLP-1R activation results in a down regulation of VTA to NAc DA signalling (Wang et al., 2015).

Whilst both optogenetics and chemogenetics have differing repertoires of advantages and disadvantages when used in isolation, together they offer a very powerful means of interrogating neural circuits and in recent papers they are used to compliment and collaborate (Li et al., 2016; Pascoli et al., 2015; Sweeney and Yang, 2015; Zhu et al., 2016).

In this chapter I establish expression of both the hM3Dq and hM4Di construct in dopamine neurons using DATCre mice. I then assess the locomotor response to CNO administration in hMDREADD expressing mice (Wang et al., 2013) and verify the change in neuron activity profile following CNO administration with *ex vivo* electrophysiology. Finally, I use the DREADD technology to test my hypothesis, generated in chapter 3, that modulation of dopamine neuron activity reduces salt consumption.

4.2. Methods

Animals

The same DATCre colony was used for all DREADD experiments as for optogenetics. Controls were wild type littermates, operated on and treated in an identical manner to the DATCre^{+} mice.

Viral vector

The DREADD construct, of human muscarinic acetylcholine receptor M3 fused to mCherry (hM3Dq-mCherry), was obtained from Prof Graeme Milligan, University of Glasgow (Armbruster et al., 2007) and was inserted into pAAV-Eifla-DIO-WPRE vectors (gift from Deisseroth Lab, Stanford: http://web.stanford.edu/group/dlab/optogenetics/sequence_info.html). DREADD construct of human muscarinic acetylcholine receptor M4 fused to mCherry (hM4Di-mCherry) was constructed according to Nawaratne et al (Nawaratne et al., 2008) and cloned into pAAV-Eifla-DIO-WPRE vectors. This was kindly done for me by Dr Choudhury. The vectors were packaged in AAV serotype 2/1 vector consisting of the AAV2 ITR genomes and the AAV1 serotype capsid gene (Vector Biolab, Philadelphia). A titre of $2.1x10^{12}$ GC/ml was used.

Surgical technique

The surgical protocol from Chapter 3 was used. Co-ordinates were AP – 3.45, ML ±0.4, DV -5.05. Following injections the wound was sutured (Mersilk, 3-0 Ethicon). Post-operative care was as previously described.

CNO dose response

Wang et al (Wang et al., 2013) showed that CNO administration (1mg/kg i.p.), in DATCre^{+} mice expressing hM3Dq resulted in hyperactivity. Wishing to minimalise hyperactivity I therefore started with a dose of 0.5mg/kg but the mice were visibly hyperactive and did not consume any jellies. Dose response in open field testing was performed 7 weeks later. All of my reported hM3Dq and hM4Di experiments used a dose of 0.05mg/kg and 0.1mg/kg respectively. The latter corresponded to the lowest effective dose used by Mahler et al for hM4Di experiments (Mahler et al., 2014).

Open field

Protocol and equipment remained unchanged from chapter 3 except that CNO or saline was injected rather than cocaine. Both the hM3Dq and hM4Di cohorts were tested after salt depletion and canteen testing during which all mice received CNO. The interval between canteen testing and open field was 7 weeks and 4 days respectively.

Salt jelly canteen testing

Post-surgery the mice were allowed to recover for 1-2 weeks then started on the handling and habituation protocol as previously described. They were salt depleted as described. On test day the mice received i.p. CNO and were placed in their testing chamber. After 30 minutes the canteen test started. Salt depletion and canteen testing was always performed on mice naïve to all testing.

Analysis of TH/mCherry co-expression

Brain tissue was processed for immunohistochemistry as previously described. Anti-TH was labelled using AlexaFluor 488. Samples from ten DATCre^{+} mice were visualised under direct fluorescent microscopy, and one section corresponding to Bregma -3.52 was identified by visualising TH only. These sections then underwent confocal laser scanning microscopy (Leica SP5 confocal microscope). AlexaFluor488 was excited by a 488-nm line of an Argon laser, mCherry by 591-nm line of a DPSS laser. Images were taken at a resolution of 1024 through objective x20／0.7 numerical aperture dry

HC Plan-Apochromat CS DIC objective (z=1.0 µm). Signal was enhanced by use of line average and frame accumulation. Acquisition of double labelling was taken sequentially.

All images were processed with Fuji software. Using TH expression, 3 areas of interest were identified, corresponding to medial, ventral and dorsolateral regions of the VTA and containing 8-13 cell bodies. mCherry images were then processed and co-expression identified.

Ex vivo electrophysiology

Preparation of acute brain slices was as previously described in Chapter 3. Specific to chemogenetic regulation, VTA dopamine neurons expressing M3 or M4 were identified by visualising mCherry. The whole-cell recording electrode (5-7 MΩ) was filled with an internal solution containing (in mM): K-Gluconate 140, KCl 5, HEPES 10, EGTA 0.1, $MgCl_2$ 2, MgATP 2, NaGTP 0.2 (pH 7.3-7.4, 280-285 mOsm). For M3, 1uM CNO (C0832, Sigma) was perfused onto the brain slice after obtaining a stable spontaneous firing for 10 minutes and change of membrane potential and firing frequency was monitored for 20 minutes. For M4, 100uM CNO was pipetted directly into the bath chamber after obtaining a stable spontaneous firing for 10 minutes. After the membrane potential was hyperpolarised, CNO was washed off with vigorous amount of aCSF.

4.3. Results

Stereotactic injection of the hM3Dq vector into the $DATCre^+$ mice led to expression of mCherry (Fig. 4.1) in 89% (95% CI 81.66 to 96.63) of TH positive neurons in the mid VTA. There was some spread to dopamine neurons in the SNc. The coordinates that I used favoured coexpression in the dorsolateral area compared to medial. *Ex vivo* electrophysiological recordings, done by Dr Tossell, showed an increase in spontaneous firing of dopamine neurons expressing mCherry followed bath application of CNO. Immunohistochemistry, showing co-expression of neurobiotin and TH, confirmed that the recorded neurons were dopaminergic.

Expression of hM3Dq in dopamine neurons resulted in a marked increase in activity following i.p. injection of CNO 0.05mg/kg. This can be seen by the open field testing, which was conducted by Dr Elaine Irvine. There is no difference between groups, with regard to the distance moved in 5 minute bins, during the 20 minute habituation period (Fig 4.2a). However, following i.p. injection, $DATCre^+$ mice that received CNO increased their activity compared to all control groups.

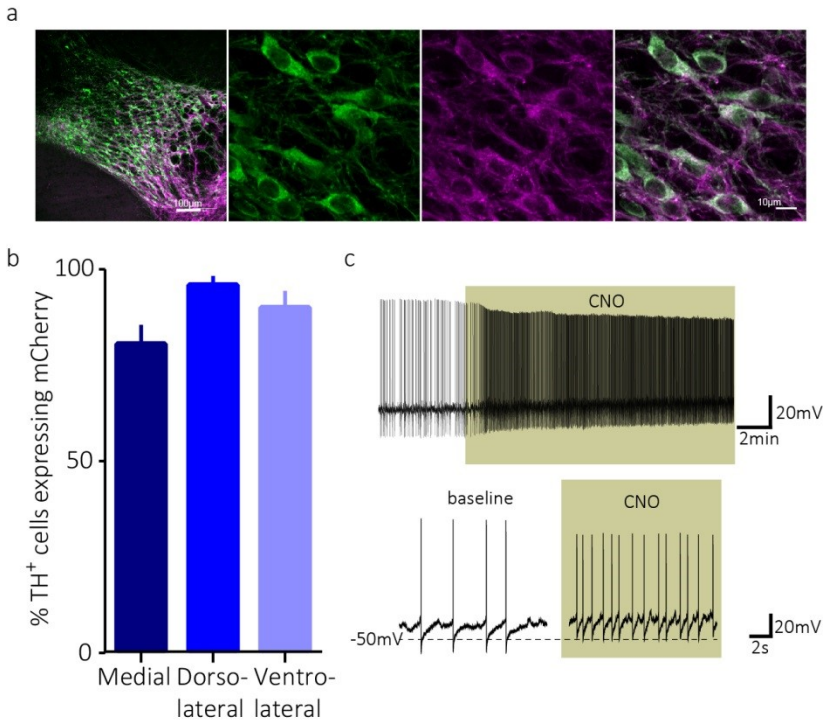

Figure 4.1: hM3Dq-mCherry expression in VTA dopamine neurons

a. Confocal images show widespread hM3Dq-mCherry expression (magenta) in TH positive cells (green) in the VTA of DATcre^{+} mice. **b.** Viral expression favoured the dorsolateral VTA rather than the medial VTA ($F(2, 24)=3.574$ $p<0.05$). **c.** Recordings of dopamine neurons in acute brain slices, bath application of CNO resulted to an increase in firing.

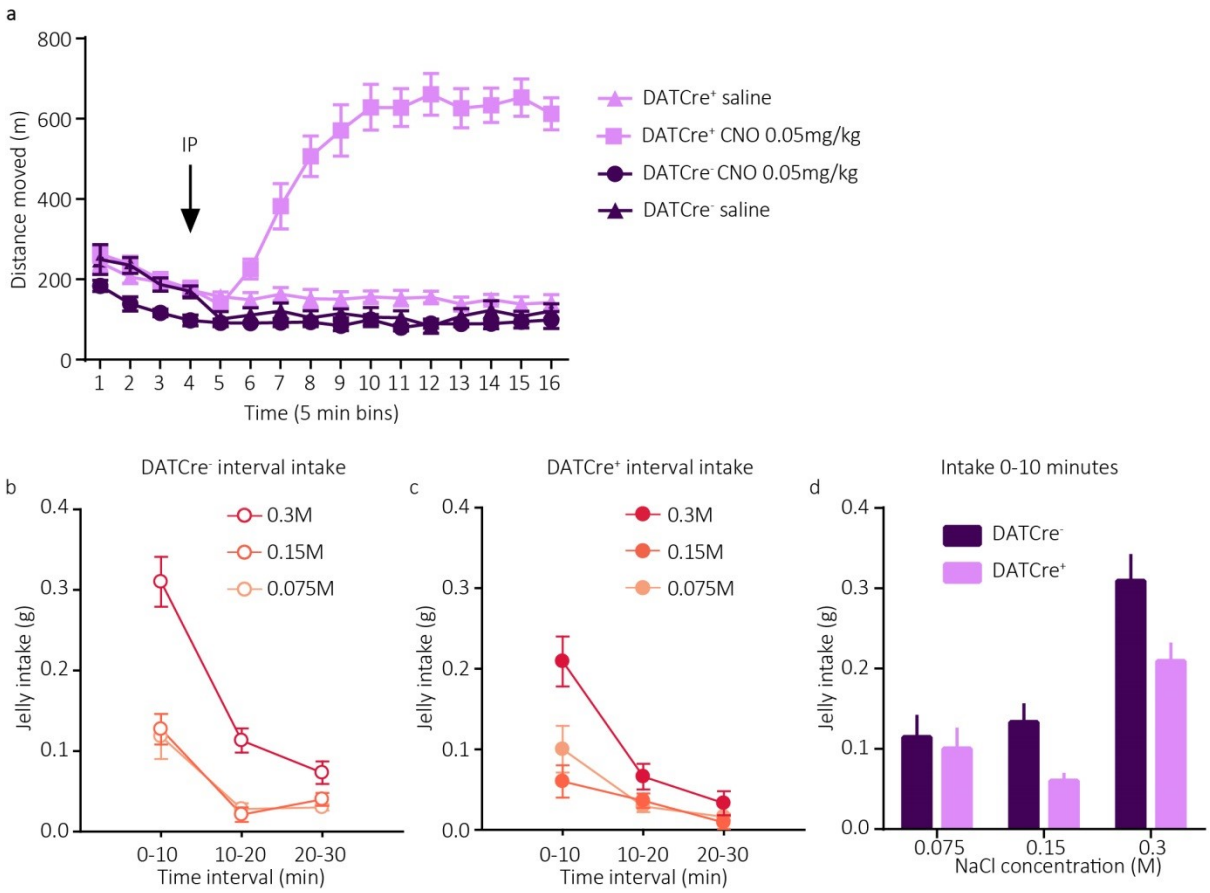

Figure 4.2: Behavioural response to chemogenetic excitation of VTA dopamine neurons

a. Following i.p. injection DATCre^{+} mice receiving CNO (n=7) increased locomotive activity in the open field testing compared to DATCre^{+} saline (n=8), DATCre^{-} CNO and DATCre^{-} saline (Genotype x Drug $F(1,23)=46.2$ $p<0.001$, Drug $F(1,23)=23.5$ $p<0.001$; Genotype $F(1,23)=71.1$ $p<0.001$). **b** and **c.** Peripheral CNO injection 30 minutes prior to the start of the salt jelly canteen test of salt-depleted mice previously injected with AAV-hM3Dq-mCherry into the VTA resulted in a significant reduction in intake across the session of DATcre^{+} mice (n=11) with respect to DATcre^{-} mice (n=12) (Genotype x Time $F(2,42)= 5.7$ $p<.01$). This reduction was not dependent on concentration (Time x Concentration x Genotype $F<1$ $p>0.5$ n.s., Concentration x Genotype $F<2.2$ $p>0.1$ n.s.). Significant differences in intake occurred primarily in the first (**d)** and last thirds of the session, pairwise comparisons; p's<0.005 for intake during both 0-10 minutes and 20-30 minutes of the session for DATcre^{+} vs DATcre^{-}.

Salt depletion and canteen testing was performed on a different cohort of mice. Administration of CNO (0.05 mg/kg) led to a reduction in intake in DATCre^{+} mice that expressed hM3Dq (Fig. 4.2b to d). The reduction, which persists across the 30 minutes was not dependent on concentration (longitudinal statistical analysis was kindly done by Dr Anushka Fernando). This result due to chemogenetic activation is different from the effect of optogenetic activation of dopamine neurons on salt seeking behaviour. Optogenetic activation led to a specific reduction in the high concentration salt jelly with no overall reduction.

I then went on to examine the effect of chemogenetic inhibition of dopamine neurons on salt seeking behaviour. Injection of the Cre dependent hM4Di-mCherry vector into the VTA resulted in widespread mCherry expression in dopamine neurons of DATCre^{+} mice (Fig. 4.3a). *Ex vivo* electrophysiological recordings showed that expression of hM4Di in dopamine neurons resulted in a reduction in activity following application of CNO.

The effect of chemogenetic inhibition of VTA dopamine neurons on locomotor activity was assessed by open field testing following the systemic administration of CNO. Results show that, following a 0.1mg/kg CNO i.p. injection, DATCre^{+} mice expressing hM4Di reduced their activity compared to controls (Fig. 4.4a). This result confirmed that the dose used was sufficient to activate the hM4Di receptor expressed in the dopamine neurons of DATCre^{+} mice and lead to a behavioural response.

When 0.01mg/kg CNO was administered i.p. to salt depleted mice, whom had previously received intra VTA injections of AAV-Eifla-DIO-hM3D(Gq)-mCherry- WPRE, testing salt seeking behaviour 30 minutes later revealed no difference in intake between DATCre^{+} and DATCre^{-} mice (Fig. 4.4 b to d). Chemogenetic inhibition of dopamine neurons, sufficient to result in a reduction in locomotor activity therefore had no effect on salt appetite. This result was consistent throughout the 30 minutes of testing and was consistent for all concentrations of salt jelly.

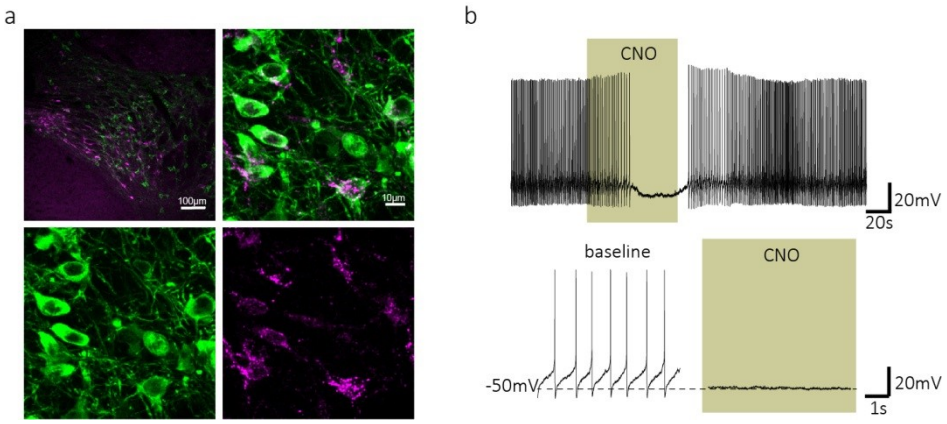

Figure 4.3: hM4Di-mCherry expression in VTA dopamine neurons

a. Confocal images show widespread hM4Di-mCherry expression (magenta) in TH positive cells (green) in the VTA of DATcre^{+} mice. **b.** Recordings of dopamine neurons in acute brain slices, bath application of CNO resulted in a decrease in firing.

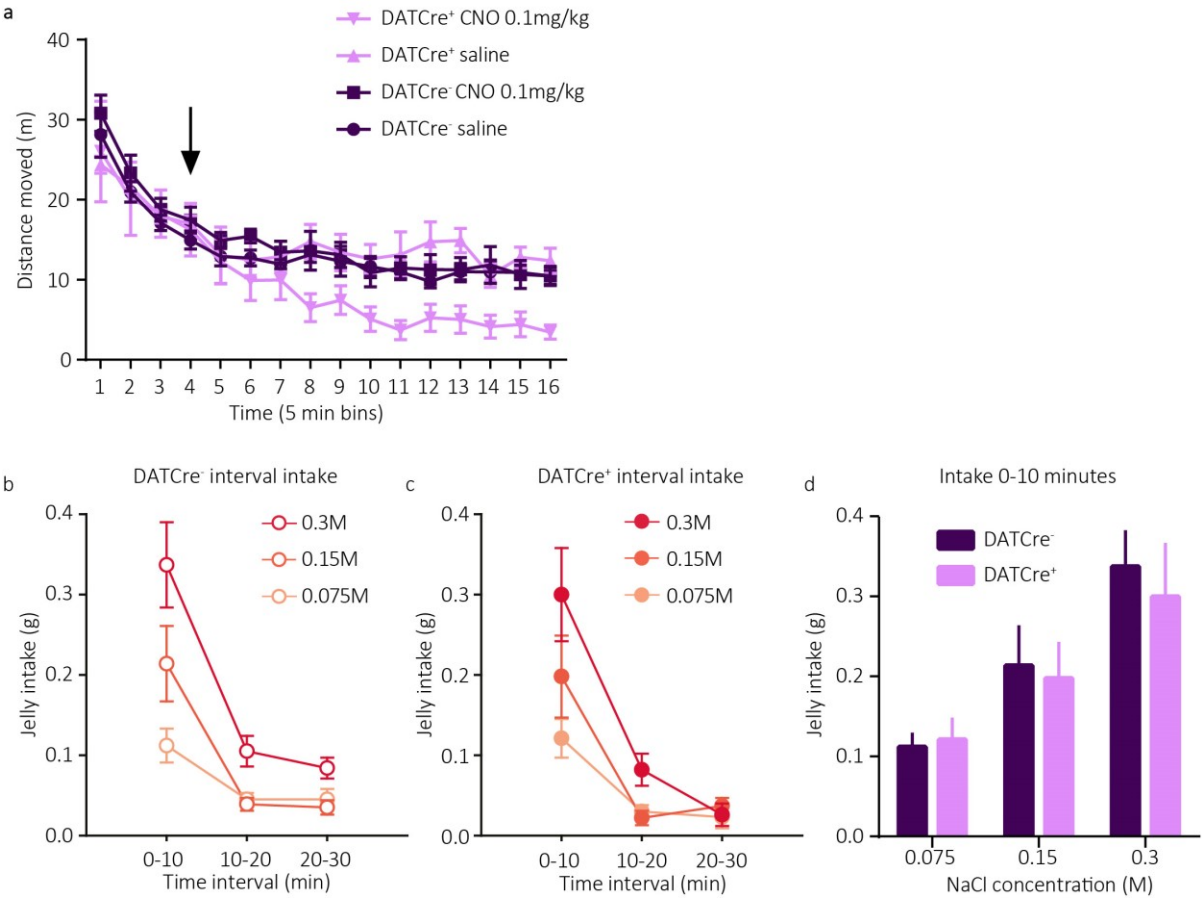

Figure 4.4: Behavioural response to chemogenetic inhibition of VTA dopamine neurons

a. Following i.p. injection DATCre^{+} mice receiving CNO (n=5) decreased locomotive activity in the open field testing compared to DATCre^{+} saline (n=8), DATCre^{-} CNO(n=6) and DATCre^{-} saline (n=6) (Treatment x Genotype $F(1,18)=8.5$ $p<.01$ pw comparisons revealed significant effects only following CNO treatment, DATcre^{+} vs DATcre^{-} $p<.005$). **b.** and **c.** Systemic CNO activation of AAV-hM4Di-mCherry prior to the salt appetite assay resulted in no significant effects on intake between genotypes DATCre^{+} (n=9) DATCre^{-} (n=11) (Time x Concentration x Genotype, Concentration x Genotype, Time x Genotype all F's <1 all p's>0.5).

4.4. Discussion

In this chapter I have shown that chemogenetic excitation of VTA dopamine neurons leads to a reduction in salt seeking behaviour. The reduction is not concentration dependent. The results therefore do not replicate those seen as a result of optogenetic stimulation of dopamine neurons; the latter is concentration dependent with only intake of the highest (0.3M NaCl) concentration jelly reduced. Chemogenetic excitation also led to an increase in locomotor activity. This is in keeping with a previous study using a higher dose of CNO (Wang et al., 2013) and might be expected following the prolonged excitation of dopamine neurons. Enhancement of locomotor activity due to high levels of dopamine has previously be shown to impair the ability of animals to complete a sequence of responses such as that required for food seeking behaviour (Lyon, 1975). This may explain the reduced intake seen with chemogenetic excitation, whilst the optogenetic study was not compromised by hyperactivity.

That my results fail to show consensus between the two methods of activity modulation, chemogenetics and optogenetics, is consistent with the literature. In the literature there are discrepancies in results between papers using the same method of activity modulation, most likely due to differences in stimulation protocols (Sternson et al., 2016b), as well as between papers using different methods of modulation (Sternson et al., 2016b; Sweeney and Yang, 2015). Two possible reasons are the difference in neuron activity intensity and pattern generated within and between chemogenetics and optogenetics, and the number and topography of neurons recruited. The *ex vivo* electrophysiology recordings show dramatic increase in neuronal firing after application of CNO. It would be expected that the *in vivo* response would be similar with unsynchronised activity. But without *in vivo* recordings it is unknown if, *in vivo*, other neuronal inputs may modulate the chemogenetically induced activity. Does the activity becomes unresponsive to physiological control so that, for example, once chemogenetic activation of VTA dopamine neurons occurs it is not possible for a reward prediction error to be processed? Different frequencies of phasic light stimulation patterns led to different levels of dopamine release in the striatum (Adamantidis et al., 2011) and there has been shown to be different behaviour effect of different patterns or stimulation, for example phasic and tonic firing (Bass et al., 2013; Chaudhury et al., 2013). Chemogenetics, with systemic administration of CNO, results in a prolonged effect on neuronal activity of all neurons expressing sufficient hM3Dq. Histochemistry revealed expression of hM3Dq

throughout the VTA and into the substantia nigra. Compared to this widespread, prolonged modulation of chemogenetics, optogenetics provides temporally discrete, synchronised activation of those neurons expressing the opsin within the illumination field of the implanted optic fibre. The blue light stimulation protocol that I used, if it was presumed to be 100% effective, would only have stimulated additional burst firing in illuminated dopamine neurons during 6% of the time. These differences between chemogenetics and optogenetics are substantial and therefore difference in behavioural response might be expected.

Dopamine neuron chemogenetic inhibition did not result in any change in salt jelly consumption, whilst optogenetic inhibition did. The dose of CNO that I used was sufficient to drive a behaviour response with regard to locomotor activity, so I would expect this discrepancy not to be due to a failure to inhibit dopamine neurons. As discussed in chapter 3, halorhodopsin inhibition has been associated with rebound excitation (Raimondo et al., 2012; Sternson et al., 2016b), the divergence in results generated by chemogenetic and optogenetic inhibition would support the view that the halorhodopsin result is due to rebound excitation rather than its inhibitory effect.

However, if optogenetic stimulation reduced salt appetite, why has inhibition had no effect? The result is consistent with previous studies showing a failure of 6-OHDA lesion and dopamine antagonism of the striatum to affect food consumption (Aberman and Salamone, 1999; Baldo et al., 2002). It may be explained in the context of the theory (discussed in Chapter 3) that salt depletion creates a withdrawal state, similar to alcohol withdrawal (Bailey et al., 2001), driven by dopamine neuron inhibition. It is this reduction in dopamine neuron activity that drives high concentration salt jelly intake. A floor effect would prevent further inhibition from exerting an effect.

There are many similarities between different appetite states. One unique property of salt appetite though, is the switch in rewarding properties of high concentration salt, from aversive to appetitive. Upon overnight fast sucrose becomes more rewarding (Domingos et al., 2011), although there is not the switch from aversion. Optogenetic excitation of dopamine neurons during salt appetite resulted in a reduction in intake of high concentration salt jelly. If this result is due to the disruption of the pathway responsible for the switch from aversive to appetitive then the effect will be unique to salt appetite. Hence there would be no effect on sucrose intake following an overnight fast. In the next chapter I therefore tested the effects of optogenetic excitation of dopamine neurons on sucrose jelly intake.

Chapter 5 Effect of optogenetic excitation of dopamine neurons on sucrose intake

5.1. Introduction

In the preceding chapters I have shown that, with regard to salt seeking behaviour, optogenetic phasic excitation of dopamine neurons results in a reduction in intake. This effect is specific for the highest concentration salt jelly. Chemogenetic excitation of dopamine neurons also reduces intake, but this effect is not concentration specific. Optogenetic inhibition of dopamine neurons led to a reduction in intake of salt jelly, which was specific for the highest concentration. However chemogenetic inhibition of dopamine neurons had no effect on intake. I was therefore interested to know if the effect of optogenetic phasic stimulation was specific to salt appetite, or whether my result is due to a more general effect on appetite. In this chapter I therefore assess the effect of optogenetic phasic excitation of dopamine neurons on intake of sucrose jellies following overnight fast.

As previously discussed, high concentration salt, in the context of salt appetite is rewarding. In a similar manner sucrose is also rewarding and there is a large literature with regard to the rewarding value of sucrose. It is "liked" and therefore elicits hedonic reaction (Grill et al., 1996) and numerous studies have used sucrose reward in fasted or food restricted animals to motivate behaviour (Adamantidis et al., 2011; Black, 1965; Hamid et al., 2016; Steinberg et al., 2013; Witten et al., 2011). Food restriction increases the "liking" and reward value of sucrose. Hedonic reaction to sucrose increases following food deprivation (Grill et al., 1996) and the preference for sucrose compared to sucralose, linked with phasic optogenetic stimulation of dopamine neurons, is increased (Domingos et al., 2011). Leptin, which is a satiety hormone, reduces the value of sucrose (Domingos et al., 2011). In a sucrose canteen setting, in rats, this is reflected in increased intake following deprivation (Lucas and Sclafani, 1988). Sucrose intake, following a fast, is therefore an experimental model which lends itself to comparison with salt appetite.

Pharmacological experiments in rats, show a role for dopamine in sucrose intake in a concentration canteen setting but there is not complete agreement. For example, Phillips *et al* (1991) showed that raclopride (D2 receptor antagonist) administration led to an increase in consumption of 34% sucrose but a reduction in 0.7% and 7% sucrose. In this experiment it was water restriction which drove consumption not food deprivation. Pardo *et al* (2015) examined the effect of dopamine

manipulations on sucrose preference but, whilst the lower concentration was freely available the higher concentration was only available after lever pressing, as a control they also tested the effect of dopamine manipulation on free consumption in a two bottle test. Pardo *et al* showed that the dopamine depleting agent tetrabenazine, a selective vesicular monoamine transporter inhibitor for VMAT-2, ecopipam (SCH39166, a D1 receptor antagonist) and haloperidol (a D2 receptor antagonist) all, dose dependently, shifted effort-related choice, decreasing lever pressing for 5% sucrose but increasing consumption of 0.3% sucrose solution (Pardo et al., 2015). However, they did not have an effect on the consumption, of either concentration, in the two bottle free consumption test. A recent optogenetic study shows that tonic activation of dopamine neuron resulted in a reduction in intake of low concentration sucrose (3%) compared to water (Mikhailova et al., 2016). This was replicated when the laser targeted dopamine terminals in the NAc. These experiments were carried out in unfasted animals. To investigate the effect of phasic optogenetic excitation of dopamine neurons on sucrose concentration intake, whilst fasted, is both interesting in the context of the above literature and as a comparison with the salt jelly results.

In this chapter I first investigate the pattern of sucrose jelly consumption in a canteen test after an overnight fast and ensure that there is no phenotype, with regard to this assay in DATCre^{+} mice. I then go on to test the effect of optogenetic excitation on sucrose intake after an overnight fast.

5.2. Methods

Mice

For the baseline sucrose jelly testing I used naïve DATCre^{+} mice on a C57Bl/6 x 129 background. All other details were the same as reported previously.

Sucrose jelly canteen testing

Jellies were made as for salt jellies but with the substitution of sucrose. For the baseline testing experiment concentrations were 5%, 10% and 20%. The concentrations were subsequently changed to 10%, 20% and 30%, to increase the canteen concentration range, and then used for the optogenetic tests. Mice were maintained on normal chow and a fasted state was achieved by the removal of food at approximately 4pm the day before testing. Testing started at 10am the following morning.

5.3. Results

The overnight fast resulted in avid intake of sucrose jellies which was not present during the unfasted, habituation period. The pattern, over time and between concentrations, resembled that for salt jellies during salt appetite. Consumption occurred predominantly in the first 10 minutes then rapidly dissipated. Intake was concentration dependent, with preference for the highest concentration (Fig. 5.1a). There was not a significant difference in intake between the lower concentration (5% and 10%) in the first 10 minutes. The concentrations, therefore, were changed for subsequent optogenetic tests. In the first 10 minutes, there was no difference in intake with regard to genotype (Fig. 5.1b).

The results from the sucrose jelly canteen test following overnight fast suggested that it was a good model to compare with salt appetite. I therefore went on to test the effect of optogenetic excitation of VTA dopamine neurons during testing. Stimulation, which began 5 minutes before testing, selectively decreased intake of the highest concentration sucrose jelly in DATCre^{+} mice compared to DATCre^{-} mice (Fig 5.2 a. to c.). This selective decrease in consumption in DATCre^{+} mice was seen in the first 10 minutes when appetite was greatest. There was no difference in intake between DATCre^{+} and DATCre^{-} mice after 10 minutes. This result mirrors the effect of optogenetic excitation of VTA dopamine neurons on salt jelly consumption in the context of salt appetite. In both, dopamine activity drives a selective decrease in the highest concentration. This suggests a common role of VTA dopamine neurons to induce avid intake of the higher concentration during intense appetite states. Optogenetic stimulation of dopamine neurons would appear to disrupt this process.

I then went on to test that this effect was attributable to optogenetic modulation of dopamine neurons by repeating the test one week later without stimulation. Surprisingly, DATCre^{+} mice selectively increased their intake of the highest concentration sucrose in the first 10 minutes compared to DATCre^{-} mice (Fig. 5.2d to e). This difference, whilst partly due to an increased intake of high concentration sucrose by DATCre^{+} mice, is in part due to a reduction in intake by DATCre^{-} mice (Fig 5.2g) on repeat testing which fails to occur in the DATCre^{+} group. The reduction in intake upon retesting DATCre^{-} mice reveals an interesting difference between sucrose and salt appetite, whilst the former shows a reduction the latter shows sensitisation.

The similar acute effect of optogenetic excitation of dopamine neurons on both sucrose and salt intake may be due to an effect on appetite but it also raises the question as to whether increased VTA dopamine activity is having no effect on appetite but merely impairing the ability of the mice to make a choice. This would result in the mice eating any jelly, whatever the concentration, thereby

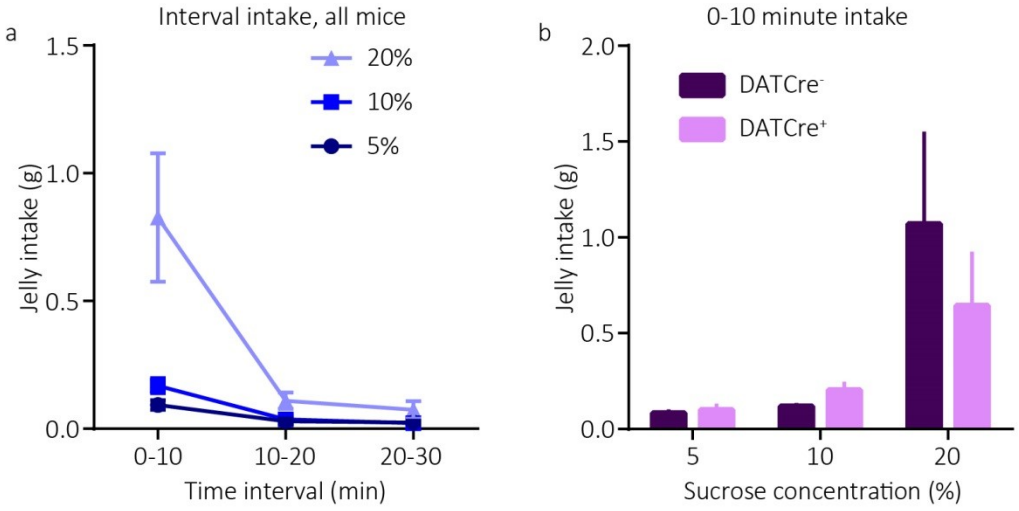

Figure 5.1: Sucrose jelly canteen intake post overnight fast

a. Following overnight fast mice show avid intake of the highest concentration (20%) sucrose. The preference for 20% dissipates after 10 minutes (Time x Concentration $F(4, 36)=6.3$ $p<0.001$, pairwise comparisons 0-10 min 20% v10% and 20% v 5% $p<0.0001$, all other comparisons ns.). **b.** In the first 10 minutes there was no significant effect of genotype on intake (genotype $F(1, 5)=0.4$ ns.).

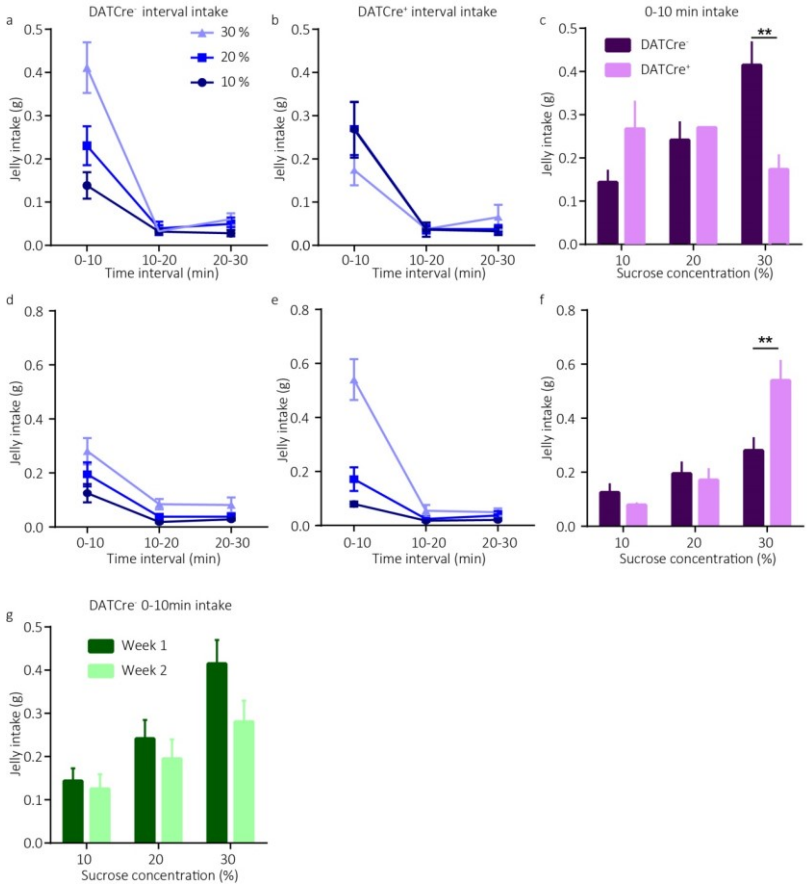

Figure 5.2: Phasic optogenetic stimulation of VTA dopamine neurons during sucrose jelly canteen

a. & **b.** Optogenetic excitation of VTA dopamine neurons resulted in a selective decrease in consumption of the highest (30%) concentration sucrose jelly in **b.** DATcre^{+} mice (n=8) with respect to **a.** DATcre^{-} mice (n=15) (Time x Conc x Genotype $F(4,84)=5.2$ $p<.005$, Conc x Genotype $F(2,42)=3.6$ $p<.05$). **c.** The selective reduction in intake of 30% sucrose jelly in DATcre^{+} mice with respect to DATcre^{-} (n=16) mice was specific to the first 10 minutes of the session (Conc x Genotype $F(2,44)=5.3$ $p<.01$, pairwise comparisons 30% sucrose DATcre^{-} vs DATcre^{+} $p<.01$). When the same mice were tested a week later without optogenetic stimulation, **e.** DATcre^{+} mice significantly increased their consumption of the 30% sucrose jelly with respect to **d.** DATcre^{-} mice (Time x Conc x Genotype $F(4,88)=10.6$ $p<.005$). **f.** The selective increase in intake of the 30% sucrose jelly by DATcre^{+} mice during the second week sucrose appetite assay occurred during the first 10 minutes of the session (Conc x Genotype $F(2,44)= 4.6$ $p<.05$. Conc $F(1.7, 37.9)=16.2$ $p<.001$, pairwise comparisons 30% sucrose DATcre^{+} (n=8) vs DATcre^{-} (n=16) $p<.01$). **g.** DATcre^{-} mice reduced sucrose intake on repeat testing (Conc x Week $F(2,30)=0.9$ ns. Conc $F(2,30)=8.1$ $p<0.005$, Week $F(1,15)=11.0$ $p<0.005$)

erasing a preference and thus generating results that appear to show a specific reduction in the normally most favoured concentration.

In the satiated state a preference for food is expressed depending on taste. Therefore the appetitive nature of a taste is not solely dependent on our physiological needs. In the salt replete state, C57Bl/6 mice express a preference for 0.075M salt (Tordoff et al., 2007). If increased VTA dopamine activity impairs the ability of mice to make a choice, I would expect optogenetic excitation of VTA dopamine neurons during a salt canteen test, in a salt replete state, to result in a selective decrease in consumption of the 0.075M NaCl jelly. Conversely, if my manipulation is a specific effect on choice during an appetite state, I would not expect a concentration specific effect since the mouse does not have an appetite. Unfortunately, as seen in chapter 2 fig.2.2c, intake of salt jellies by salt replete mice is low, it would therefore be difficult to assess the impact of optogenetic excitation of VTA dopamine neurons unless the test is run over a much longer time frame. For comparison to my previous experiments, the model required intense intake in the first 10 minutes. To drive intake I fasted the mice overnight and gave them 10% sucrose jellies. In order to test their ability to make a choice based on salt concentration I mixed the 10% sucrose with different concentrations of salt, mirroring my initial experiments.

Following overnight fast, unoperated C57BL/6, habituated according to the protocol, were given a canteen test of 10% sucrose jellies mixed with either, 0.075M, 0.15M or 0.3M salt. There was high intake in the first 10 minutes, rapidly reducing over 30 minutes. Highest intake was for 0.075M salt, least intake was for 0.3M salt (Fig. 5.3a). In my previous experimental set up, it was an appetite which was driving the concentration preference, therefore as the appetite was satiated, over the 30 minutes, the preference for high concentration reduced. In contrast, in the salty sucrose paradigm preference for the low concentration salt is stable throughout the 30 minutes (Fig. 5.3b). There was no difference between the DATCre^{+} mice and DATCre^{-} mice (Fig. 5.3c). Therefore using this new experimental model I went on to test the effect of optogenetic excitation of VTA dopamine neurons on NaCl intake in salt replete mice.

Following an overnight fast, mice which had previously received intra VTA injection of AAV-Eifla-DIO-ChR2-mCherry-WPRE and implanted with supra VTA optic fibres then habituated as protocol, underwent testing with a salty sucrose canteen. Phasic blue light illumination of the VTA, which began 5 minutes before testing, decreased intake of 10% sucrose jelly in DATCre^{+} mice compared to DATCre^{-} mice (Fig. 5.4 a and b), the effect was greatest in the first 10 minutes. In previous experiments, the decreased consumption, driven by phasic excitation dopamine neurons, of salt

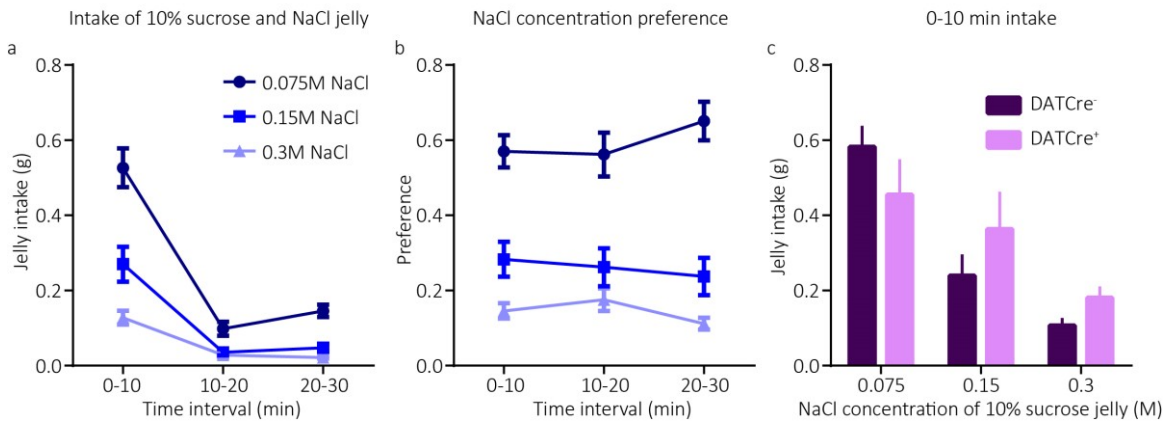

Figure 5.3: Salty sucrose jelly canteen test

a. Overnight fast generated an appetite for 10% sucrose. Intake of the lowest concentration salt was significantly greater than of the middle and of the highest concentration (Concentration x Time $F(4, 114)=12.3$ $p<0.0001$, 0-10 minutes pairwise comparisons 0.075M v 0.15M and 0.3M $p<0.0001$, 0.15M v 0.3M $p<0.001$, 20-10 minutes all pairwise comparisons ns., 20-30 minutes 0.075M v 0.15M $p<0.05$, 0.075M v 0.3M $p<0.01$). **b.** Concentration preference (intake/total interval intake) was consistent through the 30 minutes as appetite for sucrose declined (Concentration x Time $F(4, 114)=1.2$ ns., Time $F(2, 114)=2.2\times10^{-6}$ ns., Concentration $F(2, 57)=75.0$ $p<0.0001$). **c.** There was no difference in intake between $DATCre^{+}$ (n=7) mice and $DATCre^{-}$ (n=8) mice in the first 10 minutes, only the concentration was significant (Genotype x Concentration $F(2, 36)=1.9$ ns., Concentration $F(2, 36)=14.9$ $p<0.0001$, Genotype $F(1, 18)=0.4$ ns.).

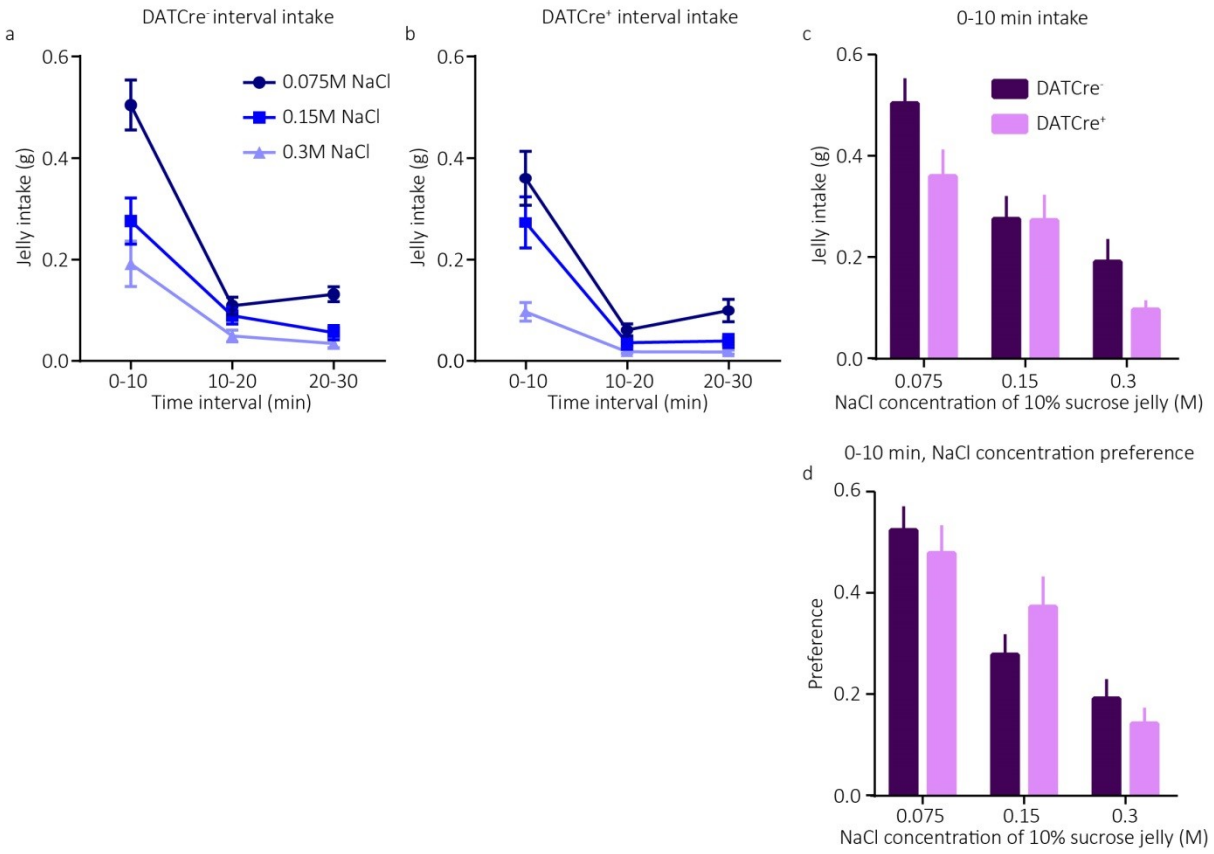

Figure 5.4: Phasic optogenetic stimulation of VTA dopamine neurons during salty sucrose jelly canteen test

Following an overnight fast optogenetic excitation of VTA dopamine neurons resulted in a significant reduction in jelly intake in **b.** DATcre^{+} (n=16) mice compared to **a.** DATcre^{-} (n=14) mice throughout the session. This reduction was not concentration specific but varied with time. (Time x Genotype x Concentration F (4,112) =1.1 p>0.3 n.s., Time x Genotype F (2, 56)=3.6 p<0.05, Genotype x Concentration F(2, 56)=0.7 ns.). **c.** In the first 10 minutes intake was reduced with regard to genotype but it was not concentration specific (Genotype x Concentration F(2, 56)=1.0 ns.). **d.** There was no difference in concentration preference between DATCre^{+} and DATCre^{-} mice (Concentration x Genotype F(2, 56)=1.0 ns., Concentration F(2, 56)=16.6 p<0.0001, Genotype F(1, 28)=1.4 ns.).

jellies, in the context of salt appetite, and sucrose jellies, after fasting, was concentration specific. The decrease consumption in salt replete mice was not concentration specific. Overall intake is reduced, however, there is no effect on the ability of the mice to make a choice based on salt concentration. This is shown by the maintenance of salt concentration preference in DATCre^{+} mice in the first 10 minutes despite increased dopamine activity (Fig. 5.4d).

5.4. Discussion

In this chapter I show that phasic excitation of VTA dopamine neurons results in a striking concentration specific reduction in sucrose intake following an overnight fast. Pharmacology studies examining the effect of dopamine on sucrose intake have been inconsistent in their results (Pardo et al., 2015; Phillips et al., 1991b, c). This may be due to the variation in sucrose concentrations used and the fact that the response appears to be concentration specific and dependent on the physiological state of the animal. Increased dopamine in the NAc, by direct infusion of nomifensine (a dopamine reuptake inhibitor), results in an increase in intake of sucrose (Hajnal and Norgren, 2001). This would seem to contradict my results but the sucrose concentration being tested was 0.3M (approx. 10%). In the sucrose canteen test optogenetic phasic excitation had no significant effect on the intake of 10% sucrose jelly, but the trend is for an increase and the study was not powered to detect this. Dopamine antagonist studies assessing intake of low concentration sucrose show either no effect for D1 receptor antagonism (Hajnal and Norgren, 2001; Pardo et al., 2015) or reduced intake (Muscat and Willner, 1989). High dose D2 receptor antagonism (sulpiride) resulted in an increase which was abrogated by coadministration of the D1 receptor antagonist (SCH23390) (Hajnal and Norgren, 2001), contrary to Muscat *et al* who showed a reduction of intake following administration of high and low dose sulpiride. High dose sucrose intake (above 30%) shows opposing results to low concentration especially when the alternative choice is low dose sucrose rather than water. SCH23390 increased the preference (but not intake) for high dose sucrose (34%)(Muscat and Willner, 1989) and sulpiride and raclopride increase preference and intake of 34% sucrose (Muscat and Willner, 1989; Phillips et al., 1991b). My experiment, by use of a concentration canteen, helps to explain some of the disagreement between the pharmacology studies.

In order to ensure that the effect of optogenetic excitation of dopamine neurons on sucrose intake was reversible, as seen with salt appetite, the mice were fasted overnight and the sucrose jelly assay performed again, but without optogenetic stimulation, one week later. This experiment shows an

interesting difference between salt appetite following salt depletion and sucrose intake following overnight fast. In chapter 2 I showed that repeated episodes of salt appetite results in sensitisation and subsequent increase in salt appetite. In comparison, repeated overnight fast resulted in the opposite effect, with a reduction in intake of sucrose during refeeding in the control group. The DATCre^{+} mice, that experienced phasic dopamine neuron excitation during the first refeed test, failed to reduce intake on the second refeed test. Phasic optogenetic excitation therefore either disrupted the normal process of habituation, as seen in controls, or resulted in the high concentration sucrose being assigned an enhanced appetitive value. The latter may not simply reflect the reinforcing ability of phasic optogenetic excitation of dopamine neurons (Adamantidis et al., 2011; Hamid et al., 2016), since in week 1, consumption of the lower concentrations was greater than that of the highest, therefore any reinforcing effect would have been expected to favour the lower concentrations.

The overnight fast/sucrose intake study was performed to compare the effect of phasic optogenetic excitation of dopamine neurons on salt seeking behaviour to its effect on sucrose seeking behaviour. The effect on sucrose seeking behaviour, following an overnight fast, was a specific reduction in intake of the highest (30%) sucrose concentration by DATCre^{+} mice compared to DATCre^{-} mice. This decrease was observed in the first 10 minutes when appetite was greatest. These results parallel those seen with optogenetic excitation of VTA dopamine neurons during salt appetite suggesting a common role of increased activity of VTA dopamine neurons to reduce intake, of the favoured concentration, during both appetite states.

Dopamine has been generally implicated in appetite and treatment for obesity previously included the use of the psychostimulant d-amphetamine (Weintraub et al., 1984), which is a broad-based reuptake inhibitor increasing serotonin, adrenaline, noradrenaline and dopamine (Sulzer et al., 1995). A number of studies have attributed the anorexic effects of amphetamine to its modulation of the dopaminergic system: dopamine antagonists (Garattini et al., 1976; Leibowitz, 1975); electrolytic lesions and 6-OHDA lesions of the nigrostriatal pathway (Carey and Goodall, 1975); (Fibiger et al., 1973) and 6-OHDA lesions of the neostriatum (Joyce and Iversen, 1984) all alleviate the hypophagic effects of amphetamine. Direct evidence of the role of dopamine in amphetamine-induced hypophagia is found in dopamine-deficient (DD) mice that are insensitive to the hypophagic effects of amphetamine (Cannon et al., 2004). Moreover, viral restoration of dopamine to the caudate-putamen of these mice reinstated amphetamine-induced hypophagia implicating dopamine signalling within the dorsal striatum in this phenomenon (Cannon et al., 2004). Too much dopamine,

due to my optogenetic manipulation may be attenuating intake in a similar manner to d-amphetamine. It has been proposed that optimal levels of dopaminergic activity are essential for the activation of motivationally-relevant behaviour (Heffner et al., 1977; Palmiter, 2007; Robbins, 2010), with an inverted U-shaped function of dopamine activity. Hence too little dopamine, exemplified in dopamine-deficient mice, inhibits feeding as these mice die of starvation unless maintained with daily injections of L-DOPA ((Szczypka et al., 1999); (Zhou and Palmiter, 1995)). Too much dopamine, as may be the case with the present optogenetic study and previous studies (Alnaser and Cooper, 1994; Scislowski et al., 1999; van der Hoek and Cooper, 1994), also results in inhibition of intake during appetite. Our optogenetic excitation protocol may therefore have resulted in dopamine activity beyond the optimal levels for engaging in food consumption during a state of appetite.

One concern of optogenetics is that it elicits non–physiological synchronous entrainment of neuron activity (Sternson et al., 2016a). Another is that the assumption that evoked behavioural changes reflect the function of the manipulated circuits. The behavioural change seen may reflect indirect effects on the independent functions of downstream circuits; these are described as off-target effects (Otchy et al., 2015). My concern was that rather than manipulating appetite I was having an effect on the ability of the mouse to express a preference, either due to an off-target effect or due to non-physiological dopamine neuron activity. I therefore tested the ability of the mice to express a preference for a salt concentration during optogenetic excitation of dopamine neurons but not in the context of a salt appetite. The experimental setup that I designed has not previously been used but it is similar to experiments on sucrose fluid intake, when the drive is water deprivation not food restriction (Pardo et al., 2015). The results showed that optogenetic excitation of dopamine neurons had no effect on need free salt concentration preference. This is very interesting since it shows that the physiological state of the mouse changes the way in which salt concentration choice is controlled by dopamine neuron activity. This reflects previous studies that have shown a difference in the response of the mesolimbic system to salt ingestion during salt depletion and when salt replete (Cone et al., 2016; Tindell et al., 2006). The result also reassures me that the concentration specific effect of optogenetic dopamine excitation on salt seeking and sucrose seeking behaviour is due to a specific effect on appetite and not the ability of the mice to express a preference. It may still be an off-target effect, replication of my experiments with pharmacological blockage of D1 or D2 receptors is needed to confirm the role of dopamine release in reducing preference for the higher concentration jelly.

In the salty sucrose experiment, there was an overall reduction in intake by DATCre^{+} mice compared to DATCre^{-} controls. This confirms that the DATCre^{+} mice did receive optogenetic stimulation of dopamine neurons, therefore the negative result on salt preference is not due to failure of the optogenetic stimulation. The result though was not anticipated since I had used 10% sucrose and in the sucrose experiment DATCre^{+} mice did not significantly reduce their intake of 10% sucrose. In the sucrose experiment 10% was the lowest concentration, and in baseline studies was least favoured. The effect of optogenetic excitation of dopamine neurons in both the salt and sucrose tests was specific to the highest, most favoured, concentration. My results therefore suggest that when only 10% sucrose is available it is most favoured, the alternative being no intake, therefore optogenetic excitation of dopamine neurons resulted in a reduction in 10% sucrose intake which would not have occured if 20% or 30% had been available. This interpretation is not consistent with previous pharmacological studies in which the effect of dopamine modulation was tested in separate 2 bottle tests comparing the intake of water to the different concentrations of sucrose. These tests showed a specific concentration effect, with raclopride reducing intake of 0.7% sucrose but increasing intake of 34% sucrose when tested separately (Phillips et al., 1991c). There are discrepancies in the few pharmacological studies that test both in the context of a canteen, separately and in a water comparison test both for sucrose (Muscat and Willner, 1989) and salt (Gilbert and Cooper, 1987a). An alternative explanation is that the addition of salt affects the way in which the sucrose concentration is perceived. Addition of salt enhances the taste of low concentration sucrose (Keast and Breslin, 2003), salty 10% sucrose is therefore not comparable to pure 10% sucrose.

My results suggest that whilst dopamine neuron activity plays a role in concentration specific intake during salt appetite, in the need-free state it does not. Pharmacological studies examining need free saline preference, using fluid restriction to drive intake, show that dopamine receptor antagonists increase saline preference especially high concentration 0.3M NaCl (Gilbert and Cooper, 1987a). A further study showed that the D1 agonist, SK&F 38395, significantly depressed choice of hypertonic saline solution, 1% and 2.5% (0.17M and 0.43M respectively), whilst the D1 antagonist, SCH 23390, increased the preference measure in the case of hypertonic saline solutions (Gilbert and Cooper, 1987b). These studies imply that dopamine plays a role in the need-free state. An alternative interpretation is that the D1 antagonist mimics reduced tonic dopamine and hence generates a state of salt appetite with an attendant rise in preference for high concentration saline. The attenuation of sucrose intake by tonic dopamine neuron activation (Mikhailova et al., 2016) was also in the unfasted state. Despite being unfasted, the baseline ingestion for the sucrose was high (>2000 lick/ 30 minutes session), this would suggest an appetite for sucrose in the unfasted state and hence a reduction in

intake following dopamine neuron activation. In contrast, in the salty sucrose experiment, the mice were salt replete and therefore had no salt appetite only a taste preference for the low concentration salt jelly when the alternative was aversive, high concentration salt.

In summary these experiments reveal similarities between salt appetite and the fasted state with respect to the impact of phasic optogenetic stimulation of dopamine neurons on intake. In both scenarios there is a specific reduction in the highest, most preferred concentration. This effect on concentration based choice is not due to an inability to express a taste preference.

Chapter 6 Discussion and future work

6.1 Conclusions

In this thesis I report my experiments exploring the role of dopamine neurons in salt seeking behaviour. I initially developed the salt jelly canteen which was able to describe salt appetite in C57/BL6 mice. Different strains of mice have different salt concentration preferences (Tordoff et al., 2007), the concentrations therefore need to be modified according to strain. My results showed that a canteen containing concentrations 0.075M, 0.15M and 0.3M salt resulted in a significant difference in intake between the concentrations in C57/BL6 mice. In the modern world there is abundance of food and therefore choice, rather than availability, is one of the main factors that control the type of food eaten. This is reflected by public health dietary initiatives which focus on encouraging healthy choices (Change 4 Life – smart swaps, NHS choices Healthy eating). A strength of my research is that a canteen assay was used and therefore the effect on choice could be assessed in addition to intake measurements.

I went on to modulate the activity of VTA dopamine neurons during salt seeking behaviour to explore the role that they play. These results showed that optogenetic excitation of VTA dopaminergic neurons specifically reduced intake of high concentration salt during salt appetite. This effect was relatively rapid, occurring within 10 minutes of stimulation, and reversible. The effect was not present a week later in the absence of excitation. The stimulation protocol was able to drive a CPP so it was not aversive, and it also did not result in a change in locomotor activity. Subsequent experiments testing the effects of optogenetic inhibition, chemogenetic excitation and chemogenetic inhibition on salt intake during salt appetite suggest that a synchronised, 'burst-like' excitation of VTA dopamine neurons is crucial to the specific reduction in intake of high concentration salt. Furthermore, I found that reduced intake during appetite was not unique to salt, as optogenetic excitation of VTA dopaminergic neurons also reduced intake of a high concentration sucrose jelly following an overnight fast. These results suggest a more general role of VTA dopamine neuron excitation in modulating intake during appetite. Importantly, the specific reduction in intake of the high concentration jelly during appetite was not due to a disruption in the ability of the mice to demonstrate a preference. Our results thus confirm a specific role of VTA dopaminergic neuron excitation in reducing intake of high concentration salt or sucrose during specific states of appetite.

The results did not support my original hypothesis which was that dopamine stimulation would increase salt intake. This led me to re-evaluate the literature especially that which examined the appetite suppressant effect of amphetamines, cocaine or dopamine receptor agonists. My results

are consistent with, and build on these reports, by showing that direct excitation of dopamine neurons can rapidly suppress intake. My results also support the theory that activation of motivated behaviour is dependent on optimal levels of dopaminergic activity (Heffner et al., 1977; Palmiter, 2007; Robbins, 2010) and demonstrates the implication of this in the context of appetite. More recent optogenetic experiments are consistent with my results by showing that dopamine neuron activation (albeit tonic not phasic), reduces intake of alcohol and sucrose (Bass et al., 2013; Mikhailova et al., 2016). My results extend on these finding since they show that there was still an appetite, since intake of the lower concentrations occurred greater than seen in salt replete mice during baseline tests (Chapter 2), and the effect is specific for the most favoured concentration. Activation of dopamine neurons is therefore attenuating the effect that an appetite has on food choice.

6.2 Clinical relevance of these findings

These results suggest that VTA dopamine neuron excitation is interfering with the process responsible for choosing the jelly with higher nutritional value during a state of appetite. The most effective management for obesity to date is Roux-en-Y bypass surgery. Many reports investigating the effect of surgery suggest that the mechanism may be due in part to changes in the gut brain axis with a subsequent change in appetite (Hansen et al., 2016; Makaronidis and Batterham, 2016). Specifically the type of food rated highly post-surgery compared to pre-surgery, and not merely the mechanical effect on gut function due to the surgery. Delineating the neural pathways involved in choice of food may enable effective treatment without the need for invasive surgery. It would also enable further research into the effect of disease, genetics and environment on this pathway.

The sight of a salt cellar, brought in from home, sitting by the lunch of a patient with diuretic resistant fluid overload,severe enough to necessitate an ITU admission, reinforces how non adherence to a low salt diet is so difficult to address, can negate the effect of a diuretic (Wilcox et al., 1983) and may result in significant morbidity and mortality. It is also often overlooked (Bowman et al., 2016). Furthering our understanding of pathological salt appetite may help to address this.

6.3 Future work

My experiments stimulate more questions than they answer. There are many basic questions to answer, for example, is there dopamine inhibition during salt appetite? Our group has started to

address this by *ex vivo* and *in vivo* electrophysiological recordings from dopamine neurons. Is the effect due to release of dopamine in the NAc or elsewhere? Will the same effect be seen with tonic excitation of dopamine neurons or is the phasic pattern of release important? Will dopamine receptor antagonists modulate the effect?

Another area of interest is how salt appetite is sated. There is rapid reduction in intake of high concentration salt jelly over the first 10 minutes. This precedes the absorption of the salt and hence any physiological correction. Satiation must therefore be by a different mechanism to induction of salt appetite. This involves a post ingestion signal since ingestion alone does not cause termination of the appetite. This is shown by persistent intake in sham feeding when a gastric fistula prevents passage of saline beyond the stomach (Roitman et al., 1997). The pattern of satiation is similar in consumption of sucrose jellies after overnight fast. It is known that in general appetite gastrointestinal hormones are responsible for satiety (Bauer et al., 2016). It is therefore likely that gastrointestinal hormones are also responsible in salt appetite. Gastrointestinal hormones that interest me, and I think warrant investigation, are guanylin and uroguanylin. Pro-peptides of guanylin and uroguanylin are released by the gut in response to a salt load and may be involved in a gut renal axis, by which ingestion of salt may lead to a natriuresis (Jose et al., 2016; Preston et al., 2012). Central expression of the uroguanylin receptors (guanylyl cyclase-C (GC-C)) is limited to VTA dopamine neurons (Gong et al., 2011). They have been investigated with regard to attention deficit disorder (Gong et al., 2011) and general appetite (Begg et al., 2014; Valentino et al., 2011) but not salt appetite. GC-C appears to be perfectly positioned to sate salt appetite.

The motivation for my research has always been the patients I treat in the course of my job as a renal and general medical registrar. It would therefore be of interest to me to move my research towards the clinical field and investigate the role of salt appetite in driving salt intake after dialysis. The first question is whether the rapid removal of sodium on dialysis triggers a salt appetite. This could to addressed by PET scanning to assess the difference in neural activity, in response to a high salt drink, for example soup, pre and post haemodialysis. The degree of salt depletion achieved during a dialysis session may be manipulated by changing the sodium concentration of the dialysate fluid. If a salt appetite is triggered the effect of drugs may be assessed. Spironolactone is a common drug, widely used in patients with heart failure and some evidence for use in dialysis patients with impaired cardiac function (Quach et al., 2016). It has an impressive effect on reducing mortality in patients taking it. The direct effect of spironolactone on salt appetite is difficult to assess due to its effect on renal salt handling. In anuric dialysis patients this confound is removed. They are therefore an ideal population to assess the importance of aldosterone in human salt appetite. ACE inhibition is

another commonly prescribed medicine, I have met one Consultant Nephrologist who continues its use in anephric patients based on anecdotal evidence that it reduces inter-dialytic weight gains, presumably through reduced salt intake. This has not been formally studied.

My opinion is that effective modification of salt intake, both on a population and patient specific basis will reduce morbidity and mortality. I hope that this thesis will contribute to the research that eventually leads to this aim.

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